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EFFECTS OF CORTICOSTEROIDS ON SERUM LIPIDS AND LIPOPROTEINS IN ADRENOCORTICAL HYPERFUNCTION

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Serum lipid and lipoprotein responses to adrenocorticosteroid therapy or excision of an adrenocortical adenoma were noted in five subjects with adrenal hyperfunction. Increase in high density ($-S_{1.21}$ 0 to 12) alpha lipoproteins was characteristic of corticosteroid administration or excess corticosteroids of endogenous (tumor) origin.

Suppression of adrenal androgen formation secondary to adrenocorticosteroid administration is an important factor in the genesis of increased levels of serum α-lipoproteins under circumstances of excessive adrenal androgen formation. Since α-lipoprotein increase also occurs in the absence of evidence of excessive adrenal androgen formation, an additional, perhaps primary, mechanism is indicated.

The response of beta ($-S_{1.21}$ 25 to 40) and lower density lipoproteins, and, hence, the response of serum lipids such as cholesterol and phospholipid appear to be subject to several factors capable of influencing the metabolism of lipids, which are modified by adrenocorticosteroid administration or excess.

DURING a study of the influence of gonadal steroids on lipid metabolism and transport[1] advantage was taken of opportunities to observe serum lipids in various adrenocortical disorders. These studies are of interest in view of the present disagreement regarding the effects of ACTH or cortisone on serum lipids and lipoproteins.[2-4]

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TABLE I. EFFECTS OF CORTICOSTEROID THERAPY OR REMOVAL OF CORTICAL ADENOMA ON PATTERNS OF SERUM LIPIDS AND LIPOPROTEINS

SUBJECT	NO. OF SERA	TOTAL CHOLESTEROL		PHOSPHOLIPID		C/P RATIO		α-LIPOPROTEINS ($-S_{1.21}$ 0-12)		β-LIPOPROTEINS ($-S_{1.21}$ 25-40)		α/β
RO (Age 6)	2	135 ±3	↑0.001*	217 ±2	↑0.05	0.63 ±0.05	ns	144 ±18	↑0.02	199 ±1	↑0.02	0.77
	8	180 ±8	↓0.001	259 ±13	↓0.01	0.70 ±0.02	ns	206 ±9	↓0.001	243 ±15	↓0.01	0.87
	11	136 ±6	↑0.01	202 ±6	↑0.01	0.68 ±0.03	ns	122 ±8	↑0.01	176 ±12	↑0.05	0.73
	4	207 ±15		298 ±23		0.70 ±0.05		229 ±25		256 ±28		0.92
AJ (Age 20)	0	—		—		—		—		—		
	6	158 ±8	↓0.01	224 ±9	↓0.05	0.71 ±0.01	ns	143 ±14	↓0.05	200 ±10	ns	0.72
	4	123 ±6	↑0.001	190 ±8	↑0.01	0.65 ±0.03	↑0.01	99 ±10	↑0.001	177 ±8	↑0.01	0.56
	4	190 ±10		227 ±4		0.93 ±0.04		188 ±8		237 ±13		0.80
GMcP (Age 36)	3	240 ±5	ns	260 ±4	ns	0.92 ±0.01	ns	196 ±11	↓0.05	320 ±12	ns	0.61
	3	247 ±4		253 ±10		0.98 ±0.02		144 ±12		290 ±11		0.50
BN (Age 36)	7	264 ±8	↑0.02	274 ±5	↑0.01	0.96 ±0.01	ns	138 ±3	↑0.001	319 ±20	↑0.0	0.44
	12	294 ±7		299 ±17		0.98 ±0.01		184 ±5		433 ±13		0.43
BH (Age 13)	8	157 ±8	ns	220 ±7	↑0.05	0.72 ±0.04	ns	205 ±10	↑0.001	167 ±8	ns	1.25
	10	173 ±3	↓0.001	243 ±6	↓0.001	0.71 ±0.01	ns	279 ±9	↓0.001	166 ±5	ns	1.69
	7	144 ±3		200 ±5		0.72 ±0.01		187 ±3		158 ±3		1.18

Mean values of lipids and lipoproteins in milligrams per cent plus or minus standard error, of urin
*The third column under each heading is the p value as determined by the "t" test. Arrow indic

SUBJECTS AND METHODS

Five women with adrenal hyperfunction were studied. Two exhibited congenital adrenal virilism with pseudohermaphroditism, one Cushing's syndrome, and two had multiple manifestations of adrenal hyperfunction in respect to glucocorticoids and androgens.

Serum lipids and lipoproteins were determined by methods previously described from this laboratory.[1]

Urinary total neutral 17-ketosteroids (17-KS) were determined by the method of Klendshoj and associates[5] and urinary 17-hydroxycorticoids (17-OHCS) by a modification of the method of Porter and Silber.[6] Urinary pregnanetriol was measured by a modification of the method of Bongiovanni and Clayton.[7] Normal values for adult women per day are: 17-KS, 6 to 14 mg.; 17-OHCS, 2.5 to 8 mg.; pregnanetriol, 0.2 to 1.8 mg.

RESULTS

Data are presented in Table I.

1. *Congenital Adrenal Virilism.*—In these two subjects the concentrations of serum alpha (high density, $-S_{1.21}$ 0 to 12) lipoproteins were low in the untreated state. The concentrations of both alpha and beta (lower density, $-S_{1.2}$

PATTERN OF SERUM LIPIDS AND LIPOPROTEINS AND ON URINARY 17-KS AND 17-OHCS

α/β LIPOPROTEIN RATIO		NO. OF STUDIES	URINARY 17-KS		NO. OF STUDIES	URINARY 17-OHCS	REMARKS
0.77 ±0.17		2	48.1 ±7.1		0	—	Congenital adrenal virilism, no therapy
	ns			↓0.001			
0.87 ±0.07		9	9.1 ±2.0		0	—	Cortison 25 to 50 mg. per day for 5 months
	ns			↑0.01			
0.73 ±0.07		4	21.3 ±2.1		1	2.7	No therapy
	ns			↓0.001			
0.92 ±0.11		4	5.3 ±1.4		3	8.4 ±4.3	Cortison 50 mg. per day for 10 weeks
—		3	70.1 ±10.6		0	—	Congenital adrenal virilism, no therapy
				↓0.001			
0.72 ±0.06		7	11.0 ±2.9		0	—	Cortison 50 to 75 mg. per day for 10 months
	ns			↑0.001			
0.56 ±0.06		5	27.5 ±2.0		0	—	No therapy
	↑0.02			↓0.001			
0.80 ±0.05		4	8.3 ±2.0		0	—	Cortisone 37.5 mg. per day for 36 months
0.61 ±0.01		4	10.5 ±2.1		0	—	Cushing's syndrome, no therapy
	↓0.02			↓0.02			
0.50 ±0.02		6	3.8 ±0.5		0	—	Postoperative state, off steroid therapy
0.44 ±0.03		5	15.0 ±0.9		4	8.0 ±2.0	Adrenal hyperfunction presumed, no therapy
	ns			↓0.001			
0.43 ±0.02		12	3.5 ±0.4		7	4.3 ±0.5	Prednisone 15 mg. per day for 2 months
1.25 ±0.09		8	23.9 ±1.7		8	13.9 ±0.8	Adrenal hyperfunctional state, no therapy
	↑0.01			↓0.01			
1.69 ±0.06		5	7.6 ±1.5		5	13.3 ±0.9	Prednisone 15 to 25 mg. per day for 4 months
	↓0.001			ns			
1.18 ±0.04		9	10.6 ±0.8		8	8.3 ±0.3	Clinical remission, 1 to 3 months after therapy

steroids in milligrams per 24 hours plus or minus standard error.
direction of change; ns means not significant, i. e., $p. > 0.05$.

25 to 40) lipoproteins and serum cholesterol and phospholipids increased significantly during oral cortisone therapy and decreased when cortisone was withdrawn. The alpha/beta lipoprotein and cholesterol/phospholipid (C/P) ratios increased consistently during treatment but the change was often not statistically significant.

Urinary 17-KS excretion was extremely high in both subjects, decreasing during therapy and increasing each time cortisone was discontinued. Urinary pregnanetriol excretion in RO was 24.7 mg. per day before treatment and 2.5 mg. per day during therapy.

2. *Cushing's Syndrome.*—This woman, with a history of Cushing's syndrome for more than two years, was studied before and after the removal of an adenoma of the left adrenal cortex. Eight months postoperatively, after all replacement cortisone therapy had been discontinued and she was clinically well, the mean α-lipoprotein concentration and the alpha/beta lipoprotein ratio were significantly less than in the preoperative state. A less marked fall in beta ($-S_{1.21}$ 25 to 40) lipoproteins and a slight increase in very low density ($-S_{1.21} > 70$)

lipoproteins were not statistically significant. Serum cholesterol and phospholipid values did not change. Urinary 17-KS excretion decreased postoperatively, in the absence of replacement therapy, to 3.8 mg. per day.

3. *Unclassified Examples of Adrenal Hyperfunction.*—Subject BN noted headache and growth of facial hair at age 29. During her only pregnancy the following year, hypertension, albuminuria, and diabetes mellitus developed, followed by truncal obesity, weakness, and bruisability. No acne, purple striae, polycythemia, or osteoporosis were noted. Menses remained regular. Bilateral enlargement of the adrenal glands was demonstrated by retroperitoneal pneumography. Urinary 17-KS and 17-OHCS excretion was normal or slightly increased.

Prior to treatment, serum cholesterol and phospholipid concentrations were elevated in association with increased concentrations of β-lipoproteins.

Urinary 17-KS and 17-OHCS excretion fell markedly during treatment with prednisone, 15 mg. daily, and lipid and lipoprotein concentrations increased without change in alpha/beta lipoprotein or cholesterol/phospholipid ratios. During the first week of therapy glycosuria increased from 7 Gm. to 27 Gm. per day. The daily dose of NPH insulin was raised from 18 to 35 units and glycosuria diminished to an average of 8 Gm. per day during the remainder of the prednisone treatment period. Comparable data on serum lipids were not obtained after prednisone withdrawal, since uncontrolled diabetes with severe retinitis developed during the ensuing 15 months. Because of these complications, nearly total bilateral adrenal resections were carried out. The adrenal tissue weighed 12.5 grams, and was histologically normal except for a small myelolipoma. During maintenance cortisone therapy postoperatively no insulin was required. This patient died at home more than a year later in acute adrenal insufficiency before medical attention could be provided.

Subject BH was studied between the ages of 13 and 15 years because of a syndrome resembling Cushing's disease, with typical fat distribution and bruisability. Blood pressure was normal. Axillary and pubic hair was profuse, breasts were undeveloped and menarche had not occurred, but the clitoris and voice were normal. Height was 55 inches, weight 120 pounds, and bone age slightly in advance of the chronologic age. The excretion of 17-OHCS and 17-KS was abnormally high. Urinary pregnanetriol excretion, determined only once prior to therapy, was within normal limits.

The patient was treated with 15 mg. prednisone daily for 6 weeks and then 25 mg. daily for an additional 12 weeks, during which time there was a significant increase in the concentration of α-lipoproteins, but no change in β-lipoproteins. Consequently, the alpha/beta lipoprotein ratio increased significantly. Serum cholesterol and phospholipid concentrations also increased. When therapy was discontinued, serum lipid and lipoprotein concentrations changed in the opposite direction.

During treatment, urinary 17-KS values fell as low as 4.5 mg. per day, but there was no significant change in the excretion of 17-OHCS. Since about 20 per cent of the administered corticosteroid may appear in the urine as Porter-Silber chromogens,[8-10] as much as 5 mg. of the observed urinary 17-OHCS may

have been of exogenous origin when the daily dose of prednisone was 25 mg. The final determination during prednisone therapy was 10.8 mg. per day, so that the amount of urinary 17-OHCS of endogenous origin may be estimated at less than 6 mg. per day. After prednisone was discontinued 17-KS excretion increased slightly to normal values and 17-OHCS excretion also increased, in comparison with treatment values when allowance for the exogenous steroid is made, to normal values.

Three months after prednisone therapy was discontinued the adrenals and ovaries were judged to be normal at laparotomy. Three months postoperatively menarche occurred at the age of 15 years, 8 months. During the subsequent two years menses were regular, the breasts developed and urinary gonadotropins were detected at low levels. The stigmata of Cushing's disease disappeared, although she grew to a height of only 56 inches since the epiphyses were nearly fused. Urinary pregnanetriol excretion remained normal. Values for the serum lipoproteins, lipids, and urinary 17-KS remained unchanged after laparotomy.

DISCUSSION

Increased levels of α-lipoproteins were associated with the administration of cortisone or prednisone, or with the presence of a relative excess of endogenous corticosteroids, as in Cushing's syndrome. Increase of α-lipoprotein levels after cortisone administration to subjects with adrenocortical hypofunction had been noted previously in this laboratory.[4]

Suppression of adrenal androgen formation by cortisone administration could be expected to lead to increased α-lipoprotein concentrations, since this is a well-recognized response of serum lipoproteins to androgen withdrawal.[1] It is noteworthy that in the subjects RO and AJ with adrenal virilism and high 17-KS excretion, cortisone therapy led to the greatest relative increases in α-lipoprotein concentrations (almost twofold increase in α-lipoproteins during the second course of therapy in each). Diminished virilism and 17-KS excretion of impressive degree also were noted. Thus it may be presumed that suppression of excessive adrenal androgen formation was responsible for the increase in α-lipoprotein concentrations noted during cortisone therapy in RO and AJ, and very likely in BH and BN as well.

In Subject GMcP with Cushing's syndrome, removal of the tumor led to serum lipoprotein changes similar to those noted when cortisone administration was discontinued in the other subjects in this study, and in previously studied subjects with adrenocortical hypofunction.[4] Since the postoperative fall in α-lipoprotein in GMcP was noted in association with markedly reduced urinary 17-KS excretion, it may be presumed that adrenal androgen release was minimal at this time. This response and the increase in α-lipoprotein which follows adrenocorticosteroid administration to subjects with adrenocortical insufficiency indicate that another factor, in addition to adrenal androgen suppression, contributes to the α-lipoprotein increase noted after adrenocorticosteroid administration. This may be a primary effect of adrenal glucocorticoids rather than a secondary effect such as adrenal androgen suppression.

Serum β-lipoprotein concentrations increased with α-lipoproteins, although not always to the same degree, in all subjects but BH in whom no change in beta levels was seen. The latter response most closely resembles that of "normal" subjects given adrenocortical steroids, as judged in the light of our previous experience.[4, 11] This suggests that in Subject BH adrenocortical function was overactive equally in respect to both corticosteroid and androgen production (possibly due to excessive ACTH release), a concept supported by her clinical course.

The relatively large increase in β-lipoprotein concentration in Subject BN with unclassified adrenal hyperfunction may have been related to the diabetes which increased in severity during treatment. The β-lipoprotein increase was of sufficient magnitude to obviate change in the alpha/beta ratio.

The serum cholesterol and phospholipid levels varied with alpha (and beta) lipoprotein levels in all subjects except GMcP with Cushing's syndrome. In this patient modest reductions in alpha and beta lipoprotein concentrations were not accompanied by any significant change in serum cholesterol and phospholipid levels because of an increase in very low density ($-S_{1.21} > 70$) lipoproteins.

Previously published reports of the effect of corticoids on serum lipids are conflicting. Adlersberg and associates[2, 12] studied normocholesterolemic patients with chronic illnesses and noted that cortisone increased cholesterol levels, associated initially with decreased but later with increased concentrations of serum neutral fat. Bunim and collaborators[13] noted that prednisolone increased serum cholesterol in five patients with arthritis, one of whom was on a constant diet. Diluzio and co-workers[14] found that adrenalectomy in dogs lowered serum cholesterol and phospholipid concentrations which were restored to normal or higher levels by cortisone therapy.

On the other hand, Perera and associates[15] reported reduction in serum cholesterol levels in two of three arthritic subjects treated with cortisone. The reduction was limited to the ester fraction and no change was noted in unesterified cholesterol levels. Similar results have not been observed by others to our knowledge. O'Connell and Burns[16] also noted decreased total cholesterol levels in two arthritic patients during cortisone administration. Decreases in serum cholesterol in two women who underwent adrenalectomy and had been treated simultaneously with hydrocortisone and monoammonium glycyrrhizinate were attributed to the administration of hydrocortisone by Elmadjian and associates.[17]

Oliver and Boyd[3, 18] administered cortisone to hypercholesterolemic men convalescent from myocardial infarctions and noted increased α-lipoprotein concentrations accompanied by lower β-lipoprotein and total cholesterol concentrations and decreased cholesterol/phospholipid ratios. Hood and Angervall's data[19] indicate an increase in serum cholesterol, caused mainly by an increase in α-lipoproteins, in a patient with adrenal hyperplasia during cortisone therapy. Thus, the data from these two laboratories and from our own are in agreement in respect to the increase in α-lipoprotein concentrations noted after the administration of cortisone or cortisone-like steroids.

Since serum cholesterol and phospholipid concentrations are determined by the concentrations of lipoproteins present in serum, the variable responses of the serum lipids to corticosteroid administration reflect the varying responses of serum beta and lower density lipoproteins to corticosteroid administration.

The effects of corticoids on the serum lipids and lipoproteins must be considered in the light of numerous and interdependent factors. The adrenal cortex produces corticoids, androgens, and estrogens—all capable of modifying serum lipoprotein metabolism—in varying amounts in health and disease. Exogenous corticoids, therefore, may influence lipoprotein metabolism by several mechanisms, e.g., by altering adrenal function through suppression of pituitary corticotropin, by a direct effect of corticoids on lipid and/or protein metabolism, or, indirectly, by influencing other metabolic parameters. The profound effects of excessive corticoids on protein, carbohydrate, and fat metabolism have been widely studied. An increased appetite for food, marked euphoria, increased physical activity, and sleeplessness may ensue and in some instances profound psychologic disturbances are noted. Redistribution of fat is a prominent clinical feature and must depend on an extensive mobilization of lipid. Although the basal metabolic rate may be increased, suppression of thyroid function has been reported.[20, 21]

The effect of dietary restriction on the response of serum lipids to corticosteroid administration has been under study in this laboratory,[4] and it has been demonstrated that the increase in serum cholesterol, phospholipids, and α-lipoproteins which may be noted during corticosteroid therapy in subjects on a constant diet may be corrected or prevented by additional calories from carbohydrates. Such additional calories reduce the weight loss and attenuate the negative nitrogen balance associated with corticosteroid therapy. The subjects in the present report, other than BN who received an 1,800 calorie "diabetic diet" during hospitalization, were not under dietary control nor did significant changes in body weight occur during therapy.

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IMPORTANCE OF AMINO ACIDS IN THE ACTIONS OF INSULIN AND SERUM SULFATION FACTOR TO STIMULATE SULFATE UPTAKE BY CARTILAGE FROM HYPOPHYSECTOMIZED RATS

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A balanced mixture of amino acids was found to act synergistically with either insulin or dialyzed serum from normal rats to increase sulfate uptake in vitro by cartilage from hypophysectomized rats. Glucose did not increase the sulfation promoting effects of insulin, the serum sulfation factor, amino acids, or the combination of amino acids and either insulin or the serum sulfation factor. Neither insulin nor the serum sulfation factor increased sulfate uptake by cartilage incubated in a medium which contained the amino acids except for valine. The serum sulfation factor was more potent than insulin in increasing cartilage sulfate uptake. In concentrations likely to be found under physiologic conditions, insulin did not have a significant effect.

SERUM from normal rats causes marked stimulation of sulfate uptake in vitro by cartilage from hypophysectomized rats.[1] This action results from the synergistic combination of a protein (sulfation factor) and dialyzable substances.[2] The protein factor is not detectable in serum from hypophysectomized rats but reappears when the animals are treated with growth hormone. Dialyzable sulfation promoting activity is found in serum from both normal and hypophysectomized rats and may be attributed partly to essential amino acids. Comparable serum components have been demonstrated in man, and recent clinical studies have suggested that assays of serum sulfation factor may be a useful index of pituitary growth hormone activity.[3]

Insulin in high concentrations also stimulates sulfate uptake in vitro by cartilage from hypophysectomized rats.[1] This effect, as well as the effect of the serum sulfation factor, has been demonstrated in incubation media which contain glucose. However, the studies reported here indicate that stimulation of sulfate uptake by cartilage in vitro by either insulin or the serum sulfation factor is not significantly altered by glucose but is profoundly influenced by amino acids.

METHODS

Animals.—Rats of the Sprague-Dawley strain* were used for all studies. For the experiments with nasal and xiphoid cartilage shown in Tables VI and VII, rats underwent hypophysectomy when they weighed about 100 grams. For the other experiments, hypophysectomy

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was performed when the rats were 21 days of age. Experiments were conducted 12 to 30 days after operation. Animals which showed unusual weight gain, gonadal enlargement, or, in the case of the larger rats, failure of the gonads to become atrophic were discarded. All animals had access to enriched white bread, Purina laboratory chow, and distilled water until the time of experiments. Hypophysectomized rats were killed by decapitation. Blood for serum from normal rats was obtained under ether anesthesia from the abdominal aorta.

Conditions of Incubations.—Techniques were modified only slightly from those of earlier reports.[1, 2] For all experiments a randomized block design was used. In certain experiments, nasal and xiphoid cartilages from each donor rat were divided into two approximately equal parts, one being assigned to treatment and the other to control group. Individual cartilage samples were placed in glass vials with 1.0 ml. of medium which contained 50 units penicillin, 50 mcg. streptomycin, and 2.5 μc S^{35}-sulfate.* The basal medium was Krebs' phosphosaline buffer,[4] pH 7.4, which had the following ion concentrations, in milliequivalents per liter: Na 158, K 4.6, Mg 2.3, Cl 123, SO_4 2.3, and (HPO_4 + H_2PO_4)40. Incubation was carried out for 24 hours at 37° C. in a metabolic incubator with shaking frequency of 120 cycles per minute. The gas phase was air.

For media which contained amino acids, the following concentrations of L-amino acids† were provided, in millimoles per liter (Eagle formula for growth of HeLa cells in tissue culture[5]): arginine 0.1, histidine 0.05, lysine 0.2, cystine 0.05, methionine 0.05, tryptophane 0.02, phenylalanine 0.1, tyrosine 0.2, isoleucine 0.2, leucine 0.2, threonine 0.2, valine 0.2, and glutamine 2.0. For certain experiments valine was omitted. Glucose, where added, was sufficient to give a final concentration of 2 mg. per milliliter.

Experiments With Insulin.—Unless otherwise indicated, the concentration of insulin was 100 mu. per milliliter.‡

In certain studies, costal cartilages were removed from hypophysectomized rats, freed of soft tissues by careful dissection, and placed in 0.9 per cent sodium chloride solution at 0 to 4° C. for 2 to 4 hours. The saline solution was changed frequently and the container was gently agitated by hand. In other studies, the cartilage samples were transferred from cold Krebs' phosphosaline buffer to buffer at 37° C. and incubated with mechanical shaking for 4 hours. The buffer solution was changed several times during this period. After these preincubations, the cartilage samples were blotted on filter paper and placed in a buffer medium with or without insulin for determination of sulfate uptake in vitro with the usual incubation technique.

Experiments With Dialyzed Serum From Normal Rats.—Serum was dialyzed with mechanical agitation in cellulose tubes for 48 hours at 3 to 4° C. against a total of 200 volumes of Krebs' phosphosaline buffer which contained no magnesium sulfate. The concentration of magnesium sulfate was restored when the incubation media were prepared. Unless otherwise indicated, the concentration of dialyzed serum in the media was 20 per cent.

Comparison of Sulfation Promoting Activities of Insulin and Dialyzed Serum From Normal Rats.—Whole serum from hypophysectomized rats was incorporated in the incubation media in a concentration of 20 per cent. The glucose content of the media was adjusted to a final concentration of 2 mg. per milliliter, and amino acids were added in the concentrations indicated previously. Dilutions of dialyzed serum from normal rats and insulin were prepared in Krebs' phosphosaline buffer.

Determination of S^{35}-Sulfate Incorporated by Cartilage.—The cartilage samples were placed in boiling water for 3 minutes, washed for 3 hours in running water, freed of any adherent soft tissue, dried, and weighed to the nearest hundredth of a milligram. Each

*Obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.

†Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

‡Glucagon-free insulin (Lot No. W-3375) was provided by Dr. W. R. Kirtley of the Clinical Research Division of Eli Lilly and Company, Indianapolis, Ind.

sample was placed in a tube with 2 ml. of concentrated hydrochloric acid plus 2 ml. of 0.3M sodium sulfate and heated in a boiling water bath for 3 hours. The sulfate was isolated as barium sulfate, plated in stainless steel cups with 4.05 cm.^{2} surface area, and counted with a thin-window Geiger-Müller tube. Uptake of S^{35}-sulfate was expressed as counts per minute per milligram of dry cartilage.

Statistical Methods.—For analysis of the results, the analysis of variance and, for calculation of regression, the method of least squares were used. Variance ratios or application of a "t" test[6] was used for the determination of p values.

RESULTS

The addition of either 100 mu. insulin per milliliter (Table I) or 20 per cent of dialyzed serum from normal rats (Table II) to a simple buffer medium produced a 25 to 50 per cent increase in sulfate uptake by costal cartilage from rats which had undergone hypophysectomy. When amino acids were added to the buffer medium, cartilage sulfate uptake was increased by 70 to 115 per cent. The combination of amino acids and either insulin or dialyzed serum from normal rats increased cartilage sulfate uptake by 200 per cent or more. These effects were not increased by the addition of glucose to the media. Furthermore, the stimulating effect of insulin on sulfate uptake by costal cartilage in a simple buffer medium without added glucose or amino acids was not abolished by previous incubation of the cartilage samples in saline at 0 to 4° C. or in buffer at 37° C. (Table III).

TABLE I. EFFECT OF INSULIN, GLUCOSE, AND AMINO ACIDS ON SULFATE UPTAKE IN VITRO BY COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS

GROUP	ADDITIONS TO INCUBATION MEDIUM	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.*	P OF DIFFERENCE
A	None	137.9 ± 9.3	B–A >0.8
B	Glucose	141.0 ± 7.6	D–C >0.6
C	Insulin	184.8 ± 10.8	C–A <0.05
D	Insulin + glucose	194.7 ± 16.1	E–A <0.001
E	Amino acids	296.6 ± 30.9	G–E <0.001
F	Amino acids + glucose	259.6 ± 19.1	E–F >0.05
G	Amino acids + insulin	448.8 ± 33.6	G–H >0.1
H	Amino acids + insulin + glucose	415.0 ± 35.0	G+A–C–E >0.001

*One costal cartilage from each of 8 rats was assigned to each treatment.

TABLE II. EFFECT OF DIALYZED SERUM FROM NORMAL RATS, GLUCOSE, AND AMINO ACIDS ON SULFATE UPTAKE IN VITRO BY COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS

GROUP	ADDITIONS TO INCUBATION MEDIUM	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.*	P OF DIFFERENCE
A	None	137.2 ± 7.7	B–A >0.7
B	Glucose	140.4 ± 8.3	D–C >0.2
C	Dialyzed NRS	204.9 ± 4.7	C–A <0.001
D	Dialyzed NRS + glucose	219.0 ± 6.7	E–A <0.001
E	Amino acids	254.7 ± 12.2	G–E <0.001
F	Amino acids + glucose	235.2 ± 13.6	E–F >0.05
G	Amino acids + dialyzed NRS	481.9 ± 15.2	G–H >0.4
H	Amino acids + dialyzed NRS + glucose	473.5 ± 19.8	G+A–C–E <0.001

NRS = normal rats' serum.
*One costal cartilage from each of 8 rats was assigned to each treatment.

TABLE III. EFFECT OF INSULIN ON SULFATE UPTAKE IN VITRO BY COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS AFTER PREINCUBATION OF CARTILAGE AT 0 TO 4° AND 37° C.

EXPERIMENT	ADDITIONS TO FINAL INCUBATION MEDIUM	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.*	P OF DIFFERENCE
(Cartilage preincubated 2 to 4 hours at 0 to 4° C.)			
	None	143.4 ± 7.2	
	Insulin	196.7 ± 12.6	<0.001
(Cartilage preincubated 4 hours at 37° C.)			
	None	153.8 ± 8.8	
	Insulin	194.6 ± 9.6	<0.001

*Eight rats in each experiment.

TABLE IV. EFFECT OF INSULIN AND OF AMINO ACIDS PLUS INSULIN ON SULFATE UPTAKE IN VITRO BY COSTAL, NASAL, AND XIPHOID CARTILAGE FROM HYPOPHYSECTOMIZED RATS

EXPERIMENT	ADDITIONS TO INCUBATION MEDIUM	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.*		
		COSTAL	NASAL	XIPHOID
1	None	202.2 ± 22.1	262.2 ± 15.9	196.8 ± 36.1
	Insulin	287.9 ± 22.9	276.4 ± 20.7	287.2 ± 30.7
	p of difference	*<0.001*	*>0.3*	*< 0.01*
2	Amino acids	308.1 ± 21.7	438.5 ± 23.3	398.8 ± 42.8
	Amino acids + insulin	596.8 ± 36.1	710.1 ± 43.6	730.2 ± 62.4
	p of difference	*<0.001*	*<0.01*	*<0.001*

*Eight rats in each experiment for costal and nasal cartilage donors, 6 for xiphoid cartilage donors.

TABLE V. EFFECT OF DIALYZED SERUM FROM NORMAL RATS AND OF AMINO ACIDS PLUS DIALYZED SERUM FROM NORMAL RATS ON SULFATE UPTAKE IN VITRO BY COSTAL, NASAL, AND XIPHOID CARTILAGE FROM HYPOPHYSECTOMIZED RATS

EXPERIMENT	ADDITIONS TO INCUBATION MEDIUM	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.*		
		COSTAL	NASAL	XIPHOID
1	None	126.1 ± 3.7	154.9 ± 9.2	104.2 ± 4.6
	Dialyzed NRS	166.3 ± 5.0	142.3 ± 5.5	155.4 ± 11.4
	p of difference	*<0.02*	*>0.05*	*<0.01*
2	Amino acids	170.4 ± 7.9	297.8 ± 7.8	159.9 ± 13.9
	Amino acids + dialyzed NRS	296.5 ± 20.8	405.7 ± 29.1	478.9 ± 39.6
	p of difference	*<0.001*	*<0.01*	*<0.001*

NRS = normal rats' serum.
*Eight rats in each experiment.

When added to a simple buffer medium, both insulin (Table IV) and dialyzed serum from normal rats (Table V) slightly increased sulfate uptake by both xiphoid and costal cartilage from hypophysectomized rats. Sulfate uptake by nasal cartilage remained unchanged by these additions. When insulin or dialyzed serum from normal rats was added to a medium which contained amino acids, the stimulating effects on sulfate uptake by costal and xiphoid cartilage were potentiated, and significant stimulation of sulfate uptake by nasal cartilage was observed. However, the addition of either insulin (Table VI) or dialyzed serum from normal rats (Table VII) to an incubation medium

which contained all the essential amino acids except valine had no effect on sulfate uptake by costal, xiphoid, or nasal cartilage.

The sulfation promoting activities of insulin and the serum sulfation factor were compared in an incubation medium which contained amino acids, glucose, and serum from rats which had undergone hypophysectomy. Highly significant linear relationships were found between the percentage increase in sulfate uptake by costal cartilage from hypophysectomized rats and the logarithm of the concentration of added insulin from 10 to 1,000 mu. per milliliter and of dialyzed serum from normal rats from 2 to 20 per cent (Fig. 1). There was a significant ($p < 0.01$) difference between the regression coefficients of 48.0 (S.D. ± 7.1) for the insulin assay and 80.5 (S.D. ± 10.3) for the serum assay. In this and other similar assays of insulin activity, the minimal detectable level of insulin was between 1 and 10 mu. per milliliter.

TABLE VI. EFFECT OF INSULIN IN A MEDIUM CONTAINING ESSENTIAL AMINO ACIDS EXCEPT VALINE ON SULFATE UPTAKE BY CARTILAGE FROM HYPOPHYSECTOMIZED RATS

EXPERIMENT	CARTILAGE (NO. OF RATS)	ADDITIONS TO INCUBATION MEDIUM	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.	P OF DIFFERENCE
1	Costal (8)	A—None	241.6 ± 16.6	B–A <0.001
		B—Insulin	348.2 ± 14.7	A–C >0.05
		C—Amino acids less valine	220.6 ± 18.1	D–C >0.5
		D—Amino acids less valine + insulin	212.9 ± 13.4	
2	Nasal (5)	Amino acids less valine	84.9 ± 9.8	
		Amino acids less valine + insulin	91.0 ± 13.7	>0.1
3	Xiphoid (4)	Amino acids less valine	86.2 ± 10.3	
		Amino acids less valine + insulin	68.2 ± 4.6	>0.2

TABLE VII. EFFECT OF DIALYZED SERUM FROM NORMAL RATS IN A MEDIUM CONTAINING ESSENTIAL AMINO ACIDS EXCEPT VALINE ON SULFATE UPTAKE BY CARTILAGE FROM HYPOPHYSECTOMIZED RATS

EXPERIMENT	CARTILAGE (NO. OF RATS)	ADDITIONS TO INCUBATION MEDIUM	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.	P OF DIFFERENCE
1	Costal (8)	A—None	180.8 ± 14.1	B–A <0.01
		B—Dialyzed NRS	251.9 ± 34.3	A–C >0.1
		C—Amino acids less valine	147.4 ± 11.6	D–C >0.3
		D—Amino acids less valine + dialyzed NRS	162.9 ± 9.3	
2	Nasal (5)	Amino acids less valine	93.4 ± 16.1	
		Amino acids less valine + dialyzed NRS	87.5 ± 8.8	>0.5
3	Xiphoid (4)	Amino acids less valine	85.7 ± 8.3	
		Amino acids less valine + dialyzed NRS	85.0 ± 5.1	>0.8

NRS = normal rats' serum.

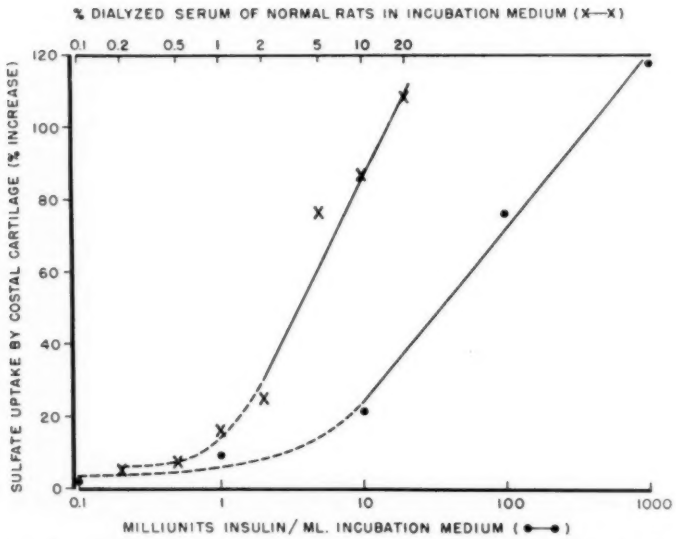

Fig. 1.—Comparison of the sulfation promoting activities of dialyzed serum from normal rats and insulin in a medium which contained essential amino acids, glucose, and serum from hypophysectomized rats. The percentage increase in sulfate uptake by costal cartilage from hypophysectomized rats which resulted from the addition of either dialyzed serum from normal rats or insulin is plotted on the ordinate. Each point represents the mean of determinations in 7 rats for the serum assay and 8 rats for the insulin assay. The regressions calculated for significant points (difference from control of $p < 0.05$) are shown by solid lines and the broken lines were drawn by inspection. For seurm 2 to 20 per cent, $Y = 80.5 \log X + 5.3$. For insulin 10 to 1000 mu. per milliliter, $Y = 48.0 \log X - 24.2$.

DISCUSSION

These results indicate that essential amino acids act synergistically with either the serum sulfation factor or insulin to stimulate uptake of sulfate in vitro by cartilage from rats which have undergone hypophysectomy. The addition of glucose to the incubation media is unnecessary to demonstrate the sulfation promoting effects of insulin, the serum sulfation factor, amino acids, or the synergistic combinations.

Previous studies have shown that the stimulation of cartilage sulfate uptake by amino acids under the conditions of these experiments is due to a balanced mixture rather than a single amino acid.[2] The omission of valine abolishes the sulfation promoting effect of the amino acid medium. The omission of threonine produces a medium which inhibits cartilage sulfate uptake. In the present studies, neither the serum sulfation factor nor insulin affected sulfate uptake by cartilage in the presence of amino acid imbalance induced by the omission of valine from the mixture of amino acids in the incubation medium.

Thus, it appears that the stimulation of cartilage sulfate uptake by either the serum sulfation factor or insulin is dependent on the amino acid pool of the tissue. The failure of either the serum sulfation factor or insulin to increase sulfate uptake by nasal cartilage without added amino acids suggests a difference between the amino acid pool of this cartilage and those of the costal and xiphoid cartilages. However, other factors may be involved since nasal cartilage appears to be less responsive to hormonal influence in vivo as well as in vitro.[7]

It was shown earlier that the amino acids used in these experiments did not completely replace the dialyzable sulfation promoting activity of serum from normal rats and that the latter activity was present also in serum from hypophysectomized rats.[2] Therefore, serum from rats which had undergone hypophysectomy was used to supplement the amino acids in a medium to compare the sulfation promoting activities of the serum sulfation factor and insulin. Although the nature of all the dialyzable sulfation promoting activity of serum remains unknown, so-called nonessential amino acids may be involved, in addition to essential amino acids. Recent studies indicate that serine is important.* It is of interest that nonessential amino acids have been found also to stimulate growth of cells in tissue culture in the presence of essential amino acids and dialyzed serum.[8]

Significant stimulation of sulfate uptake by cartilage was not found with concentrations of insulin likely to be encountered under physiologic circumstances. Although the significance of plasma insulin activity as assayed by the rat diaphragm method and other techniques remains uncertain, most workers have reported levels in plasma of adult humans after a glucose feeding equivalent to less than 1 mu. of insulin per milliliter.[9-13] In such concentrations, insulin did not significantly increase sulfate uptake by cartilage. The highest levels of plasma insulin activity have been reported by Randle,[14] who used the rat diaphragm method. Although the explanation for his high results remains obscure, he found average levels of activity equivalent to about 13 mu. of insulin per milliliter of plasma in adult human beings after a glucose feeding and comparable levels in serum from normal rats.[15] Even such levels as these could not be detected consistently by the cartilage sulfate uptake method with serum dilutions of 1:5 or greater, as used in the present experiments.

It was suggested previously that, under the conditions of these in vitro incubations, increased protein synthesis was required for increased sulfate incorporation by cartilage from rats which had undergone hypophysectomy.[2] A number of workers have shown that the chondroitin sulfate of cartilage, the form of most of the bound sulfate, occurs as a complex with a noncollagenous protein.[16-19] The experiments of Gross and collaborators[20] indicate that this complex is metabolized as a unit. Accordingly, any factor which influences protein synthesis by cartilage might be expected to affect sulfate uptake by this tissue. Thus, the actions of both the serum sulfation factor and insulin to stimulate sulfate uptake by cartilage may be indirect and primarily involve protein synthesis. Since the serum sulfation factor is dependent on growth hormone, stimulation of cartilage sulfate uptake by growth hormone in vivo may reflect the general action of growth hormone on protein synthesis.[21] The view that insulin, also, may affect protein metabolism in cartilage is consistent with the action of this hormone to increase the incorporation of amino acids into muscle protein,[22, 23] either through increased cellular transport of amino acids[24] or a direct effect on protein synthesis.[25]

*Unpublished experiments.

SUMMARY

1. A balanced mixture of amino acids was found to act synergistically with either insulin or dialyzed serum from normal rats to increase sulfate uptake in vitro by cartilage from rats which had undergone hypophysectomy.

2. Glucose did not increase the sulfation promoting effects of insulin, the serum sulfation factor, amino acids, or the combination of amino acids and either insulin or the serum sulfation factor.

3. Neither insulin nor the serum sulfation factor increased sulfate uptake by cartilage incubated in a medium which contained the amino acids except for valine.

4. The serum sulfation factor was more potent than insulin in increasing cartilage sulfate uptake. In concentrations likely to be found under physiologic conditions, insulin did not have a significant effect.

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INCREASED UPTAKE OF SULFATE BY CARTILAGE IN VITRO AFTER TREATMENT OF ALLOXAN-DIABETIC HYPOPHYSECTOMIZED RATS WITH GROWTH HORMONE

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Increased uptake of sulfate by cartilage in vitro was found after treatment with growth hormone of both diabetic and nondiabetic rats which had undergone hypophysectomy. Sulfation factor activity was demonstrated in serum obtained from both diabetic and nondiabetic hypophysectomized rats treated with growth hormone. These results indicate that the stimulation of sulfate uptake by cartilage which follows treatment of hypophysectomized rats with growth hormone is not mediated by insulin.

HYPOPHYSECTOMY in the rat markedly impairs sulfate incorporation by cartilage. Although growth hormone in vitro has little effect on sulfate uptake by cartilage, injection of growth hormone into hypophysectomized rats is followed by the appearance of a sulfation promoting factor in the serum and restoration of normal uptake of cartilage sulfate.[1]

Although insulin also has sulfation promoting activity in vitro, this hormone is less potent than the sulfation factor of serum from normal rats and, at physiologic concentrations, has little, if any, effect on sulfate uptake by cartilage from rats which have undergone hypophysectomy.[2] However, since others have reported evidence that insulin secretion is stimulated in rats by growth hormone,[3-5] the present experiments were undertaken.

In vitro uptake of sulfate has been measured in cartilage obtained from diabetic and nondiabetic hypophysectomized rats treated with growth hormone. The increased uptake of sulfate by cartilage from both diabetic and nondiabetic rats indicates that this effect of treatment with growth hormone is not mediated by insulin.

METHODS

Induction of Diabetes With Alloxan.—Male rats of the Sprague-Dawley strain underwent hypophysectomy at 21 days of age (Table I, Experiment 3 only) or when they weighed approximately 100 grams. Experiments were begun 11 to 31 days after operation. After fasting for 18 to 20 hours, the rats received intravenously, in distilled water, alloxan monohydrate,* 68 mg. per kilogram body weight. They were returned to the regular diet of enriched white bread and Purina laboratory chow and permitted to drink 0.45 per cent sodium

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*Alloxan monohydrate reagent, Nutritional Biochemicals Corporation, Cleveland, Ohio.

chloride solution. Twenty-four hours later blood was obtained from the tail vein for determination of the blood sugar by the method of Nelson.[6] Rats with a blood sugar of less than 300 mg. per 100 ml. were rejected. In the experiment in which the responses to growth hormone in diabetic and nondiabetic hypophysectomized rats were compared, all rats were maintained under comparable conditions of fasting and diet.

Treatment With Growth Hormone.—Animals were assigned in random fashion to the treatment groups. Injections of growth hormone* were given intraperitoneally in 0.9 per cent solution of sodium chloride. Control rats received an equal volume of the saline solution at the same time.

Conditions of Incubation.—Nasal, costal, and xiphoid cartilages were removed after decapitation of rats. Samples were incubated individually for 24 hours in 1.0 ml. Krebs' phosphosaline buffer which contained 50 units penicillin, 50 mcg. streptomycin, 2.5 μc S^{35}-sulfate, and 2 mg. glucose.[2]

Determination of Sulfation Promoting Activity of Serum.—Rats which served as costal cartilage donors for these experiments underwent hypophysectomy at 21 days of age and were used 12 to 30 days after operation. The glucose concentration of the test serum was determined, and the amount of glucose added to the incubation medium was adjusted to give a final concentration of 2 mg. per milliliter. The serum was incorporated in the medium in a concentration of 20 per cent, and amino acids were added in the concentrations given in the preceding report.[2]

Determination of S^{35}-Sulfate Incorporated by Cartilage.—The procedure was described in the preceding report.[2] The results from the experiments in which serum was added to the incubation medium have been corrected for differences in sulfate concentration of the various serum samples, as determined by a method previously reported.[1]

Statistical Methods.—For analysis of the results, the "t" test and the analysis of variance were used.

RESULTS

Treatment with growth hormone of hypophysectomized rats with alloxan induced diabetes increased sulfate uptake in vitro by costal and xiphoid cartilage 2 to 4 times that of cartilage from control rats (Table I). The increase was more striking in xiphoid cartilage. Although significant stimulation of sulfate uptake by nasal cartilage also resulted from treatment with growth hormone in three of the four experiments, the increase was less than that observed when costal or xiphoid cartilage was studied.

Sulfate uptake by nasal and xiphoid cartilage was significantly less in diabetic than in nondiabetic hypophysectomized rats treated with saline (Table I, Experiment 4). However, sulfate uptake by costal, nasal, and xiphoid cartilage from diabetic hypophysectomized rats treated with growth hormone did not differ significantly from uptake by cartilage from nondiabetic rats that received this treatment.

When tested in 20 per cent concentration in a medium which contained essential amino acids, serum from rats of all groups treated with growth hormone exhibited sulfation factor activity (Table II). In the four experiments, costal cartilage from nondiabetic rats which had undergone hypophysectomy incorporated 26 to 59 per cent more sulfate in serum from diabetic hypophysectomized rats treated with growth hormone than in serum from diabetic hypophysec-

*Somar, The Armour Laboratories, Lot No. R50109, provided by the Endocrinology Study Section of the National Institutes of Health.

TABLE I. EFFECT OF GROWTH HORMONE TREATMENT OF HYPOPHYSECTOMIZED RATS AND OF ALLOXAN-DIABETIC, HYPOPHYSECTOMIZED RATS ON SULFATE UPTAKE IN VITRO BY COSTAL, NASAL, AND XIPHOID CARTILAGE

EXPERIMENT	TREATMENT OF RATS	NO. OF RATS	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.		
			COSTAL	NASAL	XIPHOID
1	S, diabetic, hypox*	6	72.9 ± 1.9	130.8 ± 11.8	92.0 ± 8.6
	GH, diabetic, hypox	6	172.3 ± 14.5	167.0 ± 16.9	291.3 ± 8.1
	p of difference		*< 0.001*	*< 0.2, > 0.1*	*< 0.001*
2	S, diabetic, hypox	9	101.1 ± 5.4	132.7 ± 7.3	99.5 ± 4.2
	GH, diabetic, hypox	9	270.5 ± 18.5	222.0 ± 13.4	382.4 ± 17.4
	p of difference		*< 0.001*	*< 0.001*	*< 0.001*
3	S, diabetic, hypox	9	130.1 ± 7.7	103.1 ± 6.4	117.4 ± 11.4
	GH, diabetic, hypox	9	475.5 ± 16.7	199.6 ± 10.4	484.6 ± 27.3
	p of difference		*< 0.001*	*< 0.001*	*< 0.001*
4	S, hypox	7	78.9 ± 2.1	132.8 ± 5.2	105.1 ± 8.4
	GH, hypox	7	329.6 ± 25.3	217.1 ± 17.1	361.6 ± 16.4
	p of difference		*< 0.001*	*< 0.01*	*< 0.001*
	S, diabetic, hypox	6	77.8 ± 7.2	97.6 ± 10.2	65.4 ± 8.7
	GH, diabetic, hypox	6	265.9 ± 33.9	193.4 ± 17.5	345.2 ± 33.2
	p of difference		*< 0.001*	*< 0.001*	*< 0.001*
	S, hypox versus S, diabetic, hypox		*> 0.8*	*< 0.02*	*< 0.01*
	GH, hypox versus GH, diabetic, hypox		*> 0.1*	*> 0.3*	*> 0.5*

*S = saline; GH = growth hormone; hypox = hypophysectomized.

Average milligrams per cent blood sugar of diabetic hypophysectomized rats: Experiment 1, saline 452, growth hormone 513; Experiment 2, saline 514, growth hormone 593, Experiment 3, saline 462, growth hormone 471; Experiment 4, saline and growth hormone 568.

Rats treated with growth hormone received injections of hormone on the following schedule before being killed: Experiment 1, 1 mg. at 24 hours and 1 mg. at 12 hours; Experiment 2, 1 mg. at 48 hours, 0.5 mg. at 24 hours, and 0.5 mg. at 12 hours; Experiment 3, 1 mg. total dose in 3 injections at 42, 24, and 16 hours; Experiment 4, 1 mg. at 48 hours, 1 mg. at 24 hours, and 1 mg. at 12 hours.

TABLE II. EFFECT ON SULFATE UPTAKE BY COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS OF INCUBATION MEDIUM CONTAINING ESSENTIAL AMINO ACIDS WITH AND WITHOUT SERUM FROM DIABETIC HYPOPHYSECTOMIZED RATS TREATED WITH SALINE OR GROWTH HORMONE

EXPERIMENT	GROUP	SERUM ADDED TO INCUBATION MEDIUM*	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.	p OF DIFFERENCE
1	A	None	257.6 ± 15.0	A-B > 0.5
	B	S, diabetic, hypox rat	246.4 ± 12.9	C-B < 0.001
	C	GH, diabetic, hypox rat	390.9 ± 21.7	
2	A	None	273.6 ± 11.2	B-A < 0.05
	B	S, diabetic, hypox rat	312.8 ± 13.0	C-B < 0.001
	C	GH, diabetic, hypox rat	478.1 ± 21.8	
3	A	None	324.5 ± 23.9	B-A > 0.3
	B	S, diabetic, hypox rat	339.9 ± 20.3	C-B < 0.001
	C	GH, diabetic, hypox rat	428.6 ± 16.6	
4	A	None	340.8 ± 20.2	C-B < 0.001
	B	S, hypox rat	417.8 ± 12.9	E-D < 0.001
	C	GH, hypox rat	767.5 ± 26.7	B-D < 0.05
	D	S, diabetic, hypox rat	372.5 ± 10.1	C-E < 0.001
	E	GH, diabetic, hypox rat	525.3 ± 19.5	B-A < 0.001
				D-A > 0.1

Abbreviations same as in Table I.

*Sera were obtained from rats in corresponding experiments shown in Table I.

tomized rats treated with saline. In Experiment 4 (Table II), uptake of sulfate by costal cartilage from nondiabetic rats that had undergone hypophysectomy was 84 per cent more in serum from nondiabetic hypophysectomized rats treated with growth hormone than in serum from nondiabetic hypophysectomized rats treated with saline.

DISCUSSION

These experiments show that the presence of acute, severe insulin deficiency induced by alloxan does not prevent the increase in uptake of sulfate by cartilage which follows treatment of hypophysectomized rats with growth hormone. These studies do not, however, prove that insulin is unnecessary for metabolism of cartilage sulfate.

Schiller and Dorfman[7] have reported impairment of chondroitin sulfate synthesis in skin of rats with chronic diabetes induced by alloxan. Since glucose is the precursor of the uronic acid and hexosamine moieties of chondroitin sulfate, they postulated defective utilization of glucose to explain the impaired synthesis of chondroitin sulfate in diabetes. In the present studies, acute diabetes slightly impaired uptake of sulfate by nasal and xiphoid cartilage in hypophysectomized rats. Nevertheless, the increase in sulfate uptake by cartilage from the rats treated with growth hormone was as striking in the rats with diabetes as in the rats without diabetes.

Sulfation factor activity was demonstrated consistently in 1:5 dilutions of serum from both diabetic and nondiabetic rats treated with growth hormone. Under similar conditions in vitro, physiologic concentrations of insulin do not significantly affect sulfate uptake by cartilage from hypophysectomized rats.[2] Furthermore, others have been unable to demonstrate an increase in plasma insulin activity after treatment with growth hormone of rats this long after hypophysectomy.[3]

Although these studies provide further evidence that the serum sulfation factor is distinct from insulin, the nature of this growth hormone dependent

TABLE III. EFFECT OF GLUCAGON ON SULFATE UPTAKE IN VITRO BY COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS

EXPERIMENT	ADDITIONS TO INCUBATION MEDIUM	S^{35}-SULFATE UPTAKE (C.P.M./MG. DRY CARTILAGE) MEAN ± S.E.
1	None	244.5 ± 13.1
	Glucagon	251.7 ± 10.4
	Amino acids	514.1 ± 40.8
	Amino acids + glucagon	508.7 ± 40.7
2	Glucose	115.4 ± 9.7
	Glucose + glucagon	107.2 ± 7.8
	Glucose + amino acids	196.5 ± 17.8
	Glucose + amino acids + glucagon	167.2 ± 13.6

When additions were made, the concentration of glucagon* was 1 mcg. per milliliter, glucose 2 mg. per milliliter, and amino acids as indicated previously.[2] One costal cartilage from each of 6 rats was assigned to each treatment in Experiment 1, one from each of 8 rats to each treatment in Experiment 2. The higher counting range in Experiment 1 resulted chiefly from the use of a gas flow counter in the Geiger range to determine the radioactivity of the samples.

*Glucagon (Lot No. PA-60923) provided by Dr. W. R. Kirtley of the Clinical Research Division of Eli Lilly and Company.

factor is not clarified. Other workers have reported increased hyperglycemic factor activity in portal venous blood after treatment of rats, dogs, and cats with growth hormone.[8, 9] However, it is apparent that glucagon is not the serum sulfation factor, since this hormone has no effect on sulfate uptake in vitro by cartilage from hypophysectomized rats (Table III).

SUMMARY

1. Increased uptake of sulfate by cartilage in vitro was found after treatment with growth hormone of both diabetic and nondiabetic hypophysectomized rats.

2. Sulfation factor activity was demonstrated in serum obtained from both diabetic and nondiabetic hypophysectomized rats treated with growth hormone.

3. These results indicate that the stimulation of sulfate uptake by cartilage which follows treatment with growth hormone of rats which have undergone hypophysectomy is not mediated by insulin.

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EFFECT OF ALTERATIONS IN BLOOD pH ON DISTRIBUTION OF AMMONIA FROM BLOOD TO CEREBROSPINAL FLUID IN PATIENTS IN HEPATIC COMA

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Hepatic coma has been related to ammonia toxicity. Therefore, recent animal studies showing that a change in pH gradient can alter the distribution of ammonia between the blood stream and the brain and cerebrospinal fluid may be of therapeutic interest. Accordingly, six patients in hepatic coma and precoma were infused with 500 ml. of 0.2N hydrochloric acid and one patient, who was also acidotic, was infused with 500 ml. of 0.5N $NaHCO_3$. Their blood and cerebrospinal fluid pHs and ammonia concentrations were determined before and after the infusion which lasted 75 to 90 minutes. The pretreatment cerebrospinal fluid to arterial ammonia ratio showed no correlation with the pH gradient, since all the patients with liver disease had unusually high ratios. The arterial pH was then altered by an infusion of acid or base. The cerebrospinal fluid pH remained constant, therefore there was a change in the arterial–cerebrospinal fluid pH gradient. This was accompanied by a change in the cerebrospinal fluid to arterial ammonia ratio. When acid was infused the ratio fell in each case, and when base was infused it rose. The clinical state of the patients in precoma and coma was not significantly affected by these short term infusions.

RECENT animal studies[1] have utilized the theory of nonionic diffusion of weak acids and bases[2] to explain the method of passage of ammonia from blood to tissue and cerebrospinal fluid (CSF). Since the pK of ammonia is 8.90 at 37° C.[3] and the pH of the blood is normally about 7.40, most of the ammonia in the blood exists in the form of ammonium ion. A very small proportion is present as un-ionized or gaseous ammonia[4, 5] but only this form of ammonia can cross tissue barriers with ease.[2] This small proportion of freely diffusible ammonia can be increased or decreased by changes in blood pH.[6, 7]

When the pH of the blood is altered by an infusion of fixed acid or base, the tissue and cerebrospinal fluid pH remain relatively constant.[1, 8-11] The

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proportion of freely diffusible, un-ionized ammonia is changed on only one side of the blood–cerebrospinal fluid and blood-tissue barriers and, therefore, a redistribution of ammonia takes place.[1]

The clinical state of hepatic coma has been closely correlated with ammonia toxicity.[12-15] Therefore, attempts to change the distribution of ammonia via infusions of acid or base have been made in patients in hepatic coma, and the effect on their state of consciousness has been noted.

METHODS

The pH and ammonia concentration of femoral arterial, femoral and jugular venous blood, and cerebrospinal fluid were determined. Six patients then received infusions of 0.2N HCl (one patient received two infusions), and one patient received 0.5N $NaHCO_3$ in 500 ml. of 5 per cent dextrose. These solutions were administered through an indwelling catheter over the course of 75 to 90 minutes. Additional samples were then obtained.

It is necessary to infuse 500 ml. of 0.2N HCl or 0.5N $NaHCO_3$ within approximately 90 minutes in order to overcome the buffer capacity of the blood and the rapid respiratory compensation. Both the acidic and basic solutions may cause tissue necrosis if extravasated, and must be administered through an indwelling catheter. Weaker acid solutions (0.02N HCl) or slow infusion of strong acid have little effect on blood pH. However, if stronger acid is infused over a prolonged period of time the patient's respirations should be observed and arterial pH should be followed.

Blood and cerebrospinal fluid pH were determined with a Wynn electrode cell[16] maintained at 37° C. and a pH meter (Electronic Instruments Limited) within 15 minutes of withdrawal in oiled heparinized syringes. Duplicate determinations bracketed by standards agreed within ± 0.01 pH units. Ammonia concentrations were determined in triplicate on heparinized blood (within 15 minutes of withdrawal) according to the method of Seligson.[17]

The term, ammonia, refers to the total of both ammonium ion and un-ionized ammonia. The term C_{csf} refers to the concentration of ammonia in the cerebrospinal fluid and C_A refers to the arterial concentration. The C_{csf}/C_A refers to the distribution ratio of ammonia.* The pH gradient refers to the difference between the arterial and cerebrospinal fluid pH.

The femoral arterial (FA) whole blood ammonia concentration minus femoral or jugular venous (FV or JV) whole blood ammonia concentration (arteriovenous difference) was expressed as the ratio of the difference A-V to A times 100: $\frac{A\text{-}V \times 100}{A}$.*

RESULTS

A. *Relationship Between pH and Cerebrospinal Fluid–Arterial Ammonia Distribution in Pretreatment Patient.*—According to the theory of the nonionic diffusion of weak acids and bases,[2] ammonia, as a weak base, may be concentrated on either side of a biologic membrane depending on the direction and magnitude of the pH gradient between the two sides. Knowledge of the gradient should enable one to predict in a general way whether the ammonia concentration would be high or low on a given side of the membrane. When the arterial pH is higher than the cerebrospinal fluid pH, the cerebrospinal fluid ammonia concentration should be high in relation to that of the blood. When the arterial pH is lower than the cerebrospinal fluid pH, the cerebrospinal fluid ammonia concentration should be low in relation to that of the blood.

*The results were expressed in this manner, because during three of the infusions the patients were in a terminal state and had rising arterial ammonia concentrations.

However, patient G.H., who had a high positive arterial–cerebrospinal fluid pH gradient of + 0.11 had a low cerebrospinal fluid–arterial ammonia ratio (C_{csf}/C_A) of 0.11 (Fig. 1). J.W., with a high negative arterial–cerebrospinal fluid pH gradient of –0.13 had a high cerebrospinal fluid–arterial ammonia ratio (C_{csf}/C_A) of 0.66 (Fig. 1). These extreme examples illustrate a general lack of correlation between the ratio of the ammonia concentrations of the cerebrospinal fluid and the arterial–cerebrospinal fluid pH gradient in the pretreatment patient.

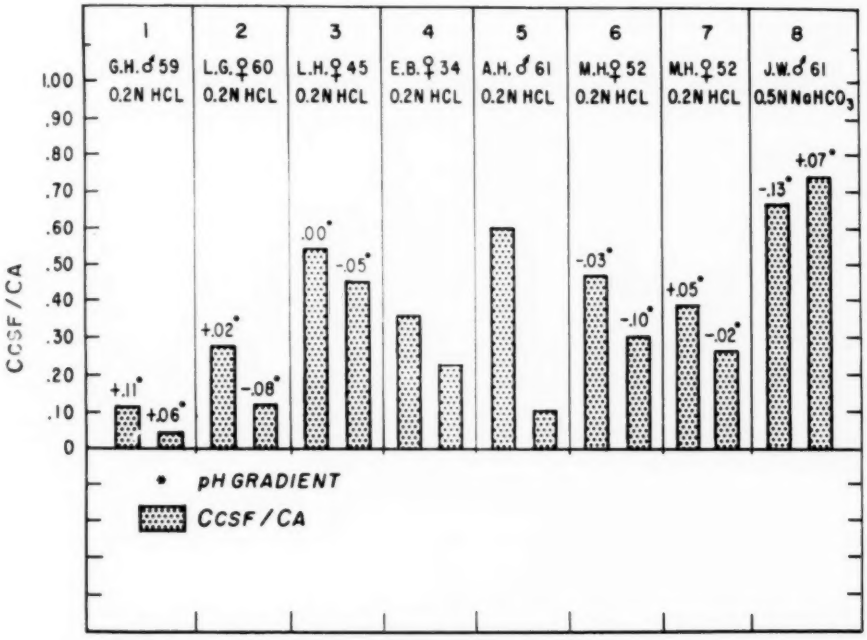

Fig. 1.—The cerebrospinal fluid–arterial ammonia ratio (C_{csf}/C_A), and arterial–cerebrospinal fluid pH gradient in seven infusions of 500 ml. of 0.2N HCl and one infusion of 500 ml. of 0.5N $NaHCO_3$ over the course of 75 to 90 minutes.

The base-line cerebrospinal fluid–arterial ammonia concentration (C_{csf}/C_A) in our patients with liver disease averaged 0.47 which compares with that of 0.58 in a large series of patients reported by McDermott.[18] The one patient we studied without primary liver disease (G.H., Fig. 1) had a ratio of 0.11 which corresponds with the average of 0.11 in McDermott's normal subjects.

B. *Cerebrospinal Fluid–Arterial Ammonia Redistribution After Infusions of Acid or Base.*—Each of the patients who received acid was either alkalotic or had a high normal blood pH prior to infusion (Table I). After the infusions of 500 ml. of 0.2N HCl the arterial pH fell from an average of 7.44 to 7.37 (Table I). Since the cerebrospinal fluid pH remained at an average of 7.41 both before and after the infusion of acid, the pH gradient was decreased in every instance. This was accompanied by an average fall in the cerebrospinal fluid–arterial ammonia ratio (C_{csf}/C_A) from 0.39 to 0.21 (Fig. 1).

The one patient who received base (J.W., Table I) had an initial arterial pH of 7.28 which rose to 7.48 after 500 ml. of 0.5N $NaHCO_3$ was administered in 80 minutes. His cerebrospinal fluid pH remained constant at 7.41, and his cerebrospinal fluid–arterial ammonia ratio (C_{csf}/C_A) rose from 0.66 to 0.73 (Fig. 1).

TABLE I. EFFECT OF INFUSION OF 500 ML. OF 0.2N HCL (CASES 1 TO 7) AND 0.5 $NaHCO_3$ (CASE 8) FOR 75 TO 90 MINUTES ON AMMONIA CONCENTRATION AND pH OF FEMORAL ARTERY (FA), FEMORAL VEIN (FV), JUGULAR VEIN (JV), AND CEREBROSPINAL FLUID (CSF)

PATIENT	SEX	AGE (YR.)	DIAGNOSIS	SITE OF SAMPLE	pH		AMMONIA (μG/ML.)	
					BEFORE INFUSION	AFTER INFUSION	BEFORE INFUSION	AFTER INFUSION
1. G. H.	M	59	Terminal carcinomatosis	FA	7.46	7.42	0.96	1.08
				FV	7.41	7.39	0.07	0.47
				JV	7.44	7.38	0.93	0.57
				CSF	7.35	7.36	0.11	0.03
2. L. G.	F	60	Biliary cirrhosis	FA	7.41	7.32	1.00	1.02
				FV	7.42	7.29	0.75	1.24
				JV	7.40	7.27	0.90	0.95
				CSF	7.39	7.40	0.27	0.10
3. L. H.	F	45	Cirrhosis, portacaval anastomosis, chronic encephalopathy	FA	7.44	7.37	1.38	1.15
				FV	7.37	7.32	0.99	0.75
				CSF	7.44	7.42	0.75	0.52
4. E. B.	F	34	Fulminant hepatitis, coma	FA			1.40	1.48
				FV			0.53	0.88
				JV			1.04	1.34
				CSF			0.50	0.31
5. A. H.	M	61	Fulminant hepatitis, precoma	FA			0.58	1.00
				FV			0.80	0.28
				JV			0.64	0.86
				CSF			0.34	0.10
6. M. H.	F	52	Laennec's cirrhosis, precoma	FA	7.42	7.33	2.60	2.50
				FV	7.41	7.32	2.20	2.30
				JV	7.38	7.26	1.96	1.94
				CSF	7.45	7.43	1.20	0.75
7. M. H.	F	52	Laennec's cirrhosis, coma	FA	7.48	7.41	1.79	2.78
				FV	7.42	7.34	0.92	0.57
				JV	7.41	7.34	1.33	0.89
				CSF	7.43	7.43	0.68	0.71
8. J. W.	M	61	Laennec's cirrhosis, bleeding varices, coma	FA	7.28	7.48	8.90	13.20
				FV	7.22	7.43	8.00	9.30
				JV	7.22	7.46	7.70	12.90
				CSF	7.41	7.41	5.90	9.58

C. *pH Changes and Arteriovenous Ammonia Differences.*—In general, the arteriovenous difference did not correlate with the pH changes and the consistent alteration in C_{csf}/C_A (Fig. 2). During three of the seven infusions of hydrochloric acid there was a decrease in the femoral arteriovenous difference (as would be predicted) but there was an increase in four cases. The jugular arteriovenous difference fell in two cases but rose in four. The patient given base (Fig. 2) had the predicted increase in femoral arteriovenous difference, but had a fall in jugular arteriovenous difference.

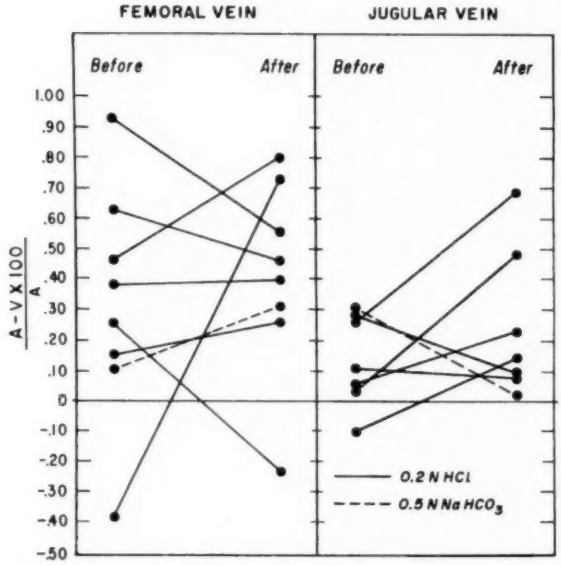

Fig. 2.—The jugular and femoral vein arteriovenous differences, $\frac{(A\text{-}V \times 100)}{A}$, before and at the termination of 75 to 90 minute infusions of 500 ml. of 0.2N HCl and 0.5N $NaHCO_3$.

D. *Effect of pH Changes on Mental State.*—There was no significant change in the mental state of any of the patients during or after infusion of acid or base.

DISCUSSION

The relation between ammonia toxicity and hepatic coma has been reviewed recently.[7] Although attempts have been made to correlate ammonia concentration with the state of consciousness of patients with liver disease by study of peripheral venous and arterial blood,[19, 20] cerebrospinal fluid,[18] and analysis of both femoral and jugular arteriovenous differences,[21-25] these have been inconclusive.

Animal experiments show that alterations in blood pH can markedly affect the distribution of infused ammonia,[1, 26] and so increase its toxicity.[7] It has been suggested that simultaneous determination of arterial pH and ammonia may give a more accurate indication of the ammonia distribution in the body.[1, 6, 26] A knowledge of the arterial–cerebrospinal fluid pH gradient should enable one to predict in a general way whether the cerebrospinal fluid–arterial ammonia ratio would be high or low.[1] Nevertheless, it was not possible to make such a prediction successfully with our patients. Prior to treatment, those with liver disease had a consistently elevated cerebrospinal fluid ammonia concentration, apparently unrelated to the pH gradient.

We confirm McDermott's finding of a high cerebrospinal fluid–arterial ammonia ratio in patients in coma, even when the arterial ammonia concentration is normal.[18] This may suggest an increase in permeability of the blood–cerebrospinal fluid barrier to ionized ammonia. However, the redistribution of ammonia with a change in pH gradient will only occur if the barrier

remains impermeable to ionized ammonia.[2] Therefore, the changes in ammonia ratio during acid or base infusion indicate no alteration in barrier permeability. High cerebrospinal fluid–arterial ammonia ratios in patients with liver disease may also be related to an increase in an ammoniacal compound in the cerebrospinal fluid such as glutamine[27-31] or an output or decreased uptake of ammonia from the cerebrospinal fluid by the brain.

In contradistinction to the base-line state of the patient, the changes in blood pH after infusion of an acid or basic solution are followed by alterations in the cerebrospinal fluid–arterial ammonia ratio in a direction which may be predicted by knowledge of the change in pH gradient. In spite of the fact that the acid infusion lasted only 75 to 90 minutes, and the average change in arterial pH was only 0.07 unit, the cerebrospinal fluid–arterial ammonia ratio changed in every case (Fig. 1). The one patient given an infusion of base showed a slight rise in his cerebrospinal fluid–arterial ammonia ratio. Animal studies have shown that these changes in the cerebrospinal fluid–arterial ammonia ratios are paralleled by changes in the ammonia ratios of the brain and muscle[1] since intracellular pH remains relatively constant during an infusion of acid or base.[10, 11]

There has been considerable and inexplicable variation in the femoral or jugular arteriovenous differences[21-25] of patients in various phases of liver disease. In this study, after an infusion of acid, arteriovenous differences showed no consistent change, although the cerebrospinal fluid–arterial ammonia ratio was altered predictably in each case. Since the arteriovenous differences were determined without concomitant blood flow studies, they might not have accurately reflected the change in tissue uptake or discharge of ammonia. The lack of correlation between jugular arteriovenous difference and the cerebrospinal fluid–arterial ammonia ratio may also be due to mixing of the cerebrovenous blood with that from other sources, and to diffusion of ammonia into and out of the spinal fluid from areas other than the choroid plexuses. In addition, the time of infusion was relatively short, and a steady state was probably not attained. Animal studies in which pH changes were of greater magnitude and steady states were achieved revealed a closer correlation among changes in pH gradient, ammonia distribution, and arteriovenous differences.[1]

After the infusion of acid there was no marked change in the mental state of any of the patients, but the majority of them were in terminal hepatic failure with deep coma and the infusion was of short duration. The administration of acid to patients in precoma may be of value since some forms of coma may possibly develop to an irreversible stage. In addition, it is felt that alkalosis in patients in precoma or coma should be corrected, and that acid infusion may be indicated when acute esophageal or gastrointestinal bleeding in patients with liver disease results in considerably elevated blood ammonia concentrations.*

*Since this study was completed, a 29-year-old patient with terminal fulminant hepatitis was placed on the artificial kidney in an attempt to lower his blood ammonia concentration. In the final hour of a 4 hour period of dialysis 220 ml. of 0.5N HCl was added to the bath. This was followed by an increase in ammonia output and an accelerated fall in the arterial ammonia concentration. On the following day 440 ml. of 0.5N HCl was added to the bath in increments from the beginning of the 4 hour period of dialysis. Although the initial arterial ammonia concentration on both days was about 4.0 γ per milliliter the total ammonia cleared on the second day was about 40 per cent more than that of the previous day.[32]

SUMMARY

1. The theory of the nonionic diffusion of weak acids and bases has been applied to a study of the effect of pH changes on ammonia distribution between blood and cerebrospinal fluid in six patients both before and after an infusion of 0.2N HCl and in one patient infused with 0.5N $NaHCO_3$. Most of the patients were in hepatic precoma or coma and their clinical state was assessed both before and after infusion.

2. The pretreatment cerebrospinal fluid–arterial ammonia ratio showed no correlation with the pH gradient. All the patients with liver disease had high ratios.

3. The arterial pH was altered by an infusion of acid or base. The cerebrospinal fluid pH remained constant, therefore changing the arterial–cerebrospinal fluid pH gradient. This was accompanied by a change in the cerebrospinal fluid–arterial ammonia ratio. When acid was infused the ratio fell in each case, and when base was infused it rose.

4. The arteriovenous differences, both jugular and femoral, showed no predictable changes on acid or alkaline infusions.

5. The clinical state of the patients in precoma or coma was not significantly affected by the infusions.

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STUDIES ON THE HEMOLYTIC PRINCIPLE OF THE FAVA BEAN

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The preparation and properties of 3 fava bean fractions is described together with studies on the effect of whole bean extract on red blood cells in the presence of serum. Hemolysis by the bean extract is inhibited by normal sera. This protection is absent in the serum of a patient with favism. The sera of the immediate family are normal in this respect although the father, without clinical evidence of favism, exhibits the same erythrocyte abnormalities as does the patient.

The importance of a serum abnormality in the causation of favism is discussed.

FAVISM is an acquired hemolytic anemia which is common in the eastern Mediterranean basin but occurs infrequently in this country as attested by the sporadic case reports appearing in the American literature.[1-3] The disease is usually associated with metabolic abnormalities of the erythrocyte, e.g., instability of glutathione and low glucose-6-phosphate dehydrogenase.[4-6] These abnormalities are not the sole factor for the reactivity of the patients to *Vicia faba*, and are not characteristic for favism alone since they occur in other forms of drug induced hemolytic anemias.[7] There are indications that susceptibility to favism is hereditary[3, 6] and is transmitted by a sex linked gene with incomplete dominance and variable expressivity.[6]

Creager and Grifford[8] described the ability of weak acacia solutions to agglutinate erythrocytes coated in vitro by a saline extract of fava beans. They suggested that this may prove useful in distinguishing erythrocytes of patients with favism from those of patients with other forms of acquired hemolytic anemia. We recently had the opportunity to observe a patient with favism and to concur tentatively with this assumption.

Our studies indicate that a plant tannin is partly responsible for results obtained in vitro. Protection afforded by normal sera against the bean extract was absent in the serum of the patient, both in remission or relapse, suggesting the importance of a serum deficiency in the causation of this disorder.

CLINICAL ABSTRACT

This 2-year-old girl of Italian extraction ate a few green fava beans and the following morning was noted to be febrile and to void dark urine. Marked pallor and scleral icterus

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were subsequently observed by the referring physician. Blood showed: red blood count, 1.2 million; hemoglobin 5.9 Gm. (37 per cent); reticulocytes 23.8 per cent; platelets 280,000, and white blood count 28,500 with 23 per cent nucleated red blood cells per 100 white blood cells. Polychromatic macrocytes and rare spherocytes were seen in the peripheral smear. Hemoglobinuria and hemosiderinuria were present. Total serum bilirubin was 2.8 mg. per cent (direct 0.35, indirect 2.45 mg. per cent). Coombs test (direct and indirect) and trypsinized erythrocyte titer were negative. Cold hemagglutinin titer was normal. Hypotonic saline fragility was 0.53 to 0.30 per cent (normal 0.44 to 0.30 per cent). Cr^{51} erythrocyte survival time was 10 days (normal 28 to 35 days). Glucose-6-phosphate dehydrogenase was 60.9 units* (normal control, 152.25 units). Glutathione was 51.5 before and 8.3 after incubation with acetylphenylhydrazine (normal control, 83 and 56, respectively).

The results of these last two tests on the parents of the patient are: mother, glucose-6-P dehydrogenase 170.56 units, glutathione 74 before and 45.5 after incubation; father, glucose-6-P dehydrogenase 35.3 units, glutathione 34 before and none after incubation.

MATERIALS AND METHODS

Preparation of the fava bean extract was performed as follows: Fresh (green) fava beans were peeled from their pods and soaked overnight in tap water. Their skins were removed and discarded. Approximately 250 Gm. of beans was immersed in 100 to 150 ml. 0.9 per cent sodium chloride and homogenized in a Waring Blendor. The resulting thick paste was squeezed through cheesecloth and the saline extract was centrifuged for 15 minutes at 12,000 r.p.m. in a Sorvall centrifuge. A small upper layer of lipid material was mixed with the clear, light green solution separated from the lower sediment. An almost imperceptible clouding of the saline extract which resulted after mixing disappeared within a few minutes. This extract was used in several experiments to homogenize another quantity of fava beans. In such instances the recovery of additional extract was poor, the final centrifugate cloudy, and a fine precipitate was noted to settle down within 30 minutes at 22° C. (supersaturation).

Ether extraction was achieved by shaking 100 ml. of the saline extract with five 20 ml. portions of ether in a separatory funnel. A brownish interfacial layer was discarded. The combined ether aliquots were evaporated by drying at low pressure and the resultant concentrate of not more than 3 ml. was mixed with 10 ml. saline. This slightly cloudy emulsion was then freed of the residual solvent by constant stirring in a water bath at 37° C.

Elution of the fava bean agglutinin from erythrocytes sensitized in vitro and all other procedures of testing, titration, sensitization, etc., were performed as described elsewhere.[8] The tannic acid used was tannin,† concentration 0.06 per cent in 0.7 per cent sodium chloride. The acacia‡ was a 5 per cent solution in isotonic saline. Since both reagents are unstable, they were freshly prepared every 24 hours. Ferric chloride was adjusted to give a final concentration of 0.5 per cent when mixed with the bean extracts. In all instances precipitation of iron tannate was followed by at least two 12 hour periods of dialysis against 0.9 per cent sodium chloride. Great care was taken to eliminate traces of $FeCl_3$ since it is also a strong agglutinin.

RESULTS

When washed normal human erythrocytes were incubated in fresh bean extracts for periods of 5 to 15 minutes, agglutination resulted upon subsequent centrifugation. These extracts had titers of 1:16 to 1:64. Addition of acacia solution, regardless of the order of mixing, enhanced the agglutinin titers to up to 1:512. After heating to 56° C. agglutination occurred only in the presence

*In remission.
†Nutritional Biochemicals Corporation.
‡Arthur H. Thomas Company.

of acacia. Boiled extracts lost all ability to sensitize. Erythrocytes of the patient with favism hemolyzed upon incubation with bean extract, 5 per cent acacia, or 0.06 per cent tannic acid, whereas normal erythrocytes did not even agglutinate in the two reagents alone.

The bean extract changed from a clear light green to a dark reddish brown color upon exposure to sunlight and air in sterile glass tubes. The maximum change required approximately 48 hours. It was greatly accelerated by shaking the solution with ether or immediately reversed by the addition of $Na_2S_2O_4$. This obviously oxidative phenomenon suggested the presence of a tannic acid since beans are rich in tannins and contact with ether is known to accelerate the formation of colored phlobophenes. There was a concomitant decrease in agglutinin titer with the change in color but undiminished activity in the presence of acacia. The addition of $FeCl_3$ to fava bean extracts resulted in the formation of a bluish black precipitate of iron tannate. The supernatant, after centrifugation and repeated dialysis, was inactive but could be restored to full agglutinin titer in the presence of acacia. No more activity could be recovered when previously aged extracts were subjected to the same treatment.

Additional observations of interest were made on the saline extracts treated with ether and on the ether fraction itself. First, the saline residue behaves exactly as an aged, $FeCl_3$ treated extract with disappearance of agglutinating activity, regardless of the presence or absence of acacia. Second, the ether soluble material is strongly hemolytic. Third, in testing the emulsions of the ether solution, the order of mixing the reagents becomes of utmost importance. The hemolytic activity of this fraction is completely abolished when added to an erythrocyte-acacia system but greatly enhanced when incubated in suboptimal concentrations with red blood cells and acacia added subsequently. That agglutination occurred in very dilute sublytic concentrations indicated that an agglutinin is present in the hemolytic emulsion. This catastoichic reaction produced a hemolysis curve which seems to rule out the presence of lysolecithin.

TABLE I. TREATMENT OF FAVA BEAN AGGLUTININ EXTRACTS WITH ACACIA AND/OR $FeCl_3$

EXTRACTS	FRESH SALINE	OLD SALINE	ETHER	ETHER RESIDUE
			hem	
Untreated	1:32 56° C.	1:1 65° C.	1:80* 80° C.	None
Untreated; with acacia	1:512 100° C.‡	1:512 100° C.	1:4096†	None
Treated with $FeCl_3$	1:2 56° C.	ϕ	1:80§ 80° C.	None
Treated; with acacia	1:512 100° C.‡	ϕ	—	—

*Hemolytic.
†Agglutinated.
‡Acacia reactive agglutinin not destroyed by aging, heating, or $FeCl_3$ (from fresh extracts only).
§Ether extractable hemolytic fraction not affected by treatment of the original saline solution with $FeCl_3$.

A summary of agglutinin titers and temperatures of inactivation of the variously treated extracts is shown in Table I.

When erythrocytes were sensitized with 0.06 per cent tannic acid and then incubated with acacia, agglutination took place. Patient's cells hemolyzed in acacia without being tanned; in fact contact with tannic acid alone produced immediate hemolysis. These erythrocytes were completely protected by 22 per cent bovine albumin or by any normal, type-compatible plasma or serum. Of greatest importance was the absence of such protection by patient's own plasma or serum. It is also interesting to note that normal erythrocytes presensitized with an Rh antibody or with agglutinins eluted from erythrocytes of patients with idiopathic acquired hemolytic anemias were agglutinated but never hemolyzed in 0.06 per cent tannic acid solution. Furthermore, the agglutinating substances which could be eluted with ease from normal erythrocytes sensitized in vitro by bean extract could never be demonstrated in eluates obtained by the same technique from patient's erythrocytes.

DISCUSSION

The diagnosis of favism is usually made on the basis of clinical and hematologic findings of a hemolytic anemia of acute onset, subsequent to the ingestion of raw, or partially cooked fava beans. The erythrocytes of these patients show abnormal glutathione patterns and low glucose-6-phosphate dehydrogenase activity. It has been mentioned that these findings do not always correlate with the in vivo hemolytic process, nor are they restricted to individuals susceptible to fava beans. The patient's father, for instance, who is not known to be sensitive, has an erythrocyte defect similar to that of his daughter (see values in the Clinical Abstract). However, his serum, and also the mother's serum can protect normal erythrocytes from sensitization by bean extract, whereas the serum of the patient does not have this effect. This may afford an explanation why hemolysis never developed in the father upon numerous ingestions of fava beans although he shares the same erythrocyte abnormality with his daughter.

Red blood cells from patients with favism and those incubated with the bean extract are identical in that they react to acacia solutions. This reagent appears therefore to be a useful and simple adjunct to distinguish affected red blood cells. However, the hemolytic principle can be eluted from in vitro coated erythrocytes but not from the cells of the patient. One may be tempted to speculate that the amounts of coating substances vary widely in these two instances. It seems more plausible that the fractions that can be demonstrated in vitro in the fava bean extract are not present in the patient.

The hemolytic principle obtained in vitro from the bean extract depends in part upon a heat-labile tannin (complex). This has been suggested by loss of agglutinating ability after oxidation. No attempt has been made to test recovery of activity after reversal of this phenomenon by sodium hydrosulfite, since it was felt that the system is already artificial without the introduction of a reducing agent. Additional confirmation of the presence of an active tannin

was obtained by ferric chloride precipitation. Here again, loss of activity followed the removal of the bluish black precipitate. The other two components of the hemolytic principle are an acacia reactive, heat-stable fraction in the untreated fresh, old, and $FeCl_3$ treated fresh extracts, and an ether extractable hemolysin, not precipitated by ferric chloride and only partly affected by heat. It is insoluble but easily emulsified in saline. It may be argued that the latter is but the saline fraction itself, highly concentrated and enhanced in its activity by acacia (agglutination titer to 1:4,000). Against this argument one has the behavior of the saline soluble bean concentrate which is heat labile and has never been shown to be hemolytic.

It has been found that red blood cells from persons susceptible to favism have, in most instances, a normal survival time when injected into normal recipients who subsequently ingest beans.[7, 10, 11] Primaquine-sensitized red blood cells behave differently since they hemolyze in compatible recipients upon administration of the drug.[12] It has also been shown that the erythrocyte abnormalities associated with favism are not characteristic for the disease. Because of this, the importance of a serum deficiency in favism becomes obvious. It may prove a diagnostic test for determination of susceptibility, and may serve to throw light on the genetic aspects of this disease. The prophylactic administration of plasma or a plasma fraction for the prevention of hemolysis is an additional possibility which would merit investigation.

SUMMARY

Studies were performed on the hemolytic principle of fava bean extracts. The results were compared with observations made on erythrocytes of a patient with favism. The following observations have been made:

1. The bean extract contains a saline soluble, heat-labile agglutinin, probably a plant tannin complex, a saline soluble, heat-stable agglutinin reacting in the presence of acacia, and a partly heat-stable, ether soluble hemolysin.

2. Agglutinating substances can always be eluted from normal erythrocytes sensitized in vitro by the bean extract. No such substances could be eluted from patient's erythrocytes sensitized in vivo.

3. Normal plasma or serum will protect erythrocytes from the sensitizing ability of bean extract. Patient's plasma or serum never had this effect.

The suggestion has been made that this serum deficiency, if established as a common characteristic, would greatly facilitate the study of susceptibility and inheritance of this disorder.

We are grateful to Dr. Harald A. Wurzel for his interest and cooperation.

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GAMMA GLOBULIN IN EXPERIMENTAL STAPHYLOCOCCAL INFECTIONS

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In the treatment of staphylococcal infections in man the main difficulty is that of overcoming, or preventing the development of, sizeable local foci of infection. In studying possible protective factors a system was chosen which reproduces at least some of the features of this sort of infection in man. In this investigation it is shown that systemic administration of gamma globulin is ineffective in modifying the development of local staphylococcal lesions in the skin of guinea pigs and does not prolong the period of time during which an antibiotic can affect the course of these lesions.

Gamma globulin contains a small quantity of staphylococcal antitoxin. It is suggested that the reported protective effects of gamma globulin in acute lethal infections in mice may be attributable to its content of antitoxin; some evidence for this view is given.

Gamma globulin is ineffective in the prevention or treatment of focal staphylococcal lesions.

IN RECENT years staphylococcal infections have aroused widespread interest partly because of their frequency and variety, and partly because of the difficulties encountered in their treatment. These difficulties in treatment are caused by a number of features of staphylococcal infection and are by no means confined to problems created by resistance to the common antibiotic drugs. For this reason it is natural that workers have sought for methods, other than administration of antibiotic drugs and surgical drainage, which might have a beneficial effect on the course of these infections. In recent years several workers have reported that human gamma globulin, given parenterally, has a favorable effect on the course of experimental and human disease caused by staphylococci and by other bacteria. The observations in humans[1-3] are difficult to assess because of the variable course of the diseases studied and the variety of treatments received by the patients in addition to the gamma globulin. The studies of gamma globulin in bacterial infection in laboratory animals[4-7] have been concerned with acute lethal infections in mice, the organisms being given in one series of observations by intracerebral inoculation and in all the others by intraperitoneal injection.

In studying possible protective factors in staphylococcal infection it is reasonable to choose an experimental system which reproduces as far as possible the important features of this sort of infection in man. Disease caused by

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staphylococci in humans is only rarely acute and fulminant in its course. The main difficulty in the management of staphylococcal infection is in overcoming local foci and in preventing new primary or metastatic foci from becoming established. For this reason it seemed worth studying the effect of gamma globulin on a local focus of staphylococcal infection in the skin, one of the most common sites of staphylococcal disease. In particular, it was believed important to determine whether treatment with gamma globulin could make it more difficult for a local focus of infection to become established. A method by which this could be done was already available since Miles and his colleagues[8] had devised a quantitative technique for the evaluation of local infections in the course of their extensive work on defense reactions of the skin. An analysis of the action of gamma globulin when this technique is used is presented here, and a number of experiments in which acute infections in mice were induced are also described.

MATERIALS AND METHODS

Staphylococcus aureus, strain W81, was used in the experiments on guinea pig skin. This strain is of the "epidemic" bacteriophage type 80/81 and was originally isolated in a nursery epidemic in Winnipeg. It has been studied by Dr. J. C. Colbeck, who sent a specimen to this laboratory. Preliminary experiments with eight strains showed that W81 gave the best dose response line and it was of interest that this strain is strongly coagulase-positive in guinea pig plasma. A dried stock was prepared by the gelatin ascorbic acid method of Stamp (1947) and a tube was taken up and grown on plain agar slopes once every 2 weeks. For the guinea pig experiments the organisms were grown in nutrient broth for 16 to 20 hours at 37° C.

Staph. aureus, strain Giorgio, was also used in a number of experiments with guinea pigs, and strain Smith was used for the infections in mice.

Guinea pigs were female albinos of the Hartley strain, weight limits 350 to 450 grams. *Mice* were Carworth Farm male albino mice of strain CFW, weight limits 18 to 22 grams.

Human gamma globulin was used in the form of poliomyelitis immune globulin (Lederle). This preparation contains 16.5 (±1.5) per cent gamma globulin and 1:10,000 sodium ethyl mercurithiosalicylate as preservative. Staphylococcus antitoxin (globulin modified) from the Connaught Medical Research Laboratories, Toronto, which contains approximately 2,000 units per milliliter. Staphylococcus alpha toxin from strain Wood 46 (Lederle) was used. Erythromycin as Erythrocin lactobionate (Abbott) was used. Chloramphenicol as Chloromycetin (Parke, Davis) was given intramuscularly.

Guinea Pig Experiments.—The method closely followed that described by Miles and associates.[8] Guinea pigs were clipped and depilated with a mixture of barium sulfide and Tide made up as a smooth paste in 10 per cent glycerol in water. The bacterial suspension was centrifuged and the deposit was resuspended in physiologic saline. Serial fivefold or half log dilutions were made in saline and were injected intradermally in doses of 0.1 ml. In most experiments two series of dilutions were used, one in saline and the other in saline-adrenaline solution to give 2 mcg. adrenaline in each intradermal dose. Each guinea pig received 10 to 20 injections and the sites were chosen at random in relation to the suspensions injected. Each suspension was injected at a total of 4 to 10 sites. The diameter of the erythematous and indurated area at each injection site was measured 24 to 48 hours after injection. The observer was unaware both of the suspension injected into the particular site and, when a systemic agent had been administered, which guinea pigs had received it and which were controls.

RESULTS

It was found that Miles' results could be reproduced and that with doses of bacteria which resulted in lesions of 5 to 15 mm. in diameter, the mean diameter of the lesions was directly related to the log dose of bacteria. As with other experimental staphylococcal infections the dose of bacteria necessary to initiate an infection was large and the actual number of bacteria per dose was usually 5×10^6 to 1×10^8 in the range of doses used. The smaller lesions were indurated and erythematous plaques; the larger lesions and sometimes the smaller ones contained pus and often showed a central area of necrosis. Heat-killed suspensions of the same doses of bacteria gave no lesions or, at most, 1 to 2 mm. of erythema. None of the lesions became chronic; after about 48 hours they tended slowly to resolve, leaving indurated plaques of inflammation which persisted for a considerable time.

Effect of Systemic Gamma Globulin on Staphylococcal Skin Lesions.—In seven experiments guinea pigs were given an intraperitoneal injection of gamma globulin at various times from 3 to 24 hours before initiation of the intradermal infection. The control animals in each experiment received injections of saline. The dose of gamma globulin varied between 0.2 ml. and 1.0 ml., and was 0.5 ml. in four of the experiments. In Fig. 1 two representative experiments are shown. It can be seen that the lesions, both in the saline and the adrenaline series, are the same size in the pigs which received gamma globulin as in those which did not. Moreover, no qualitative difference could be detected in the appearance of the lesions or the timing of their development.

Effect of Gamma Globulin in Association With Erythromycin on Staphylococcal Skin Lesions.—Although gamma globulin alone did not influence the

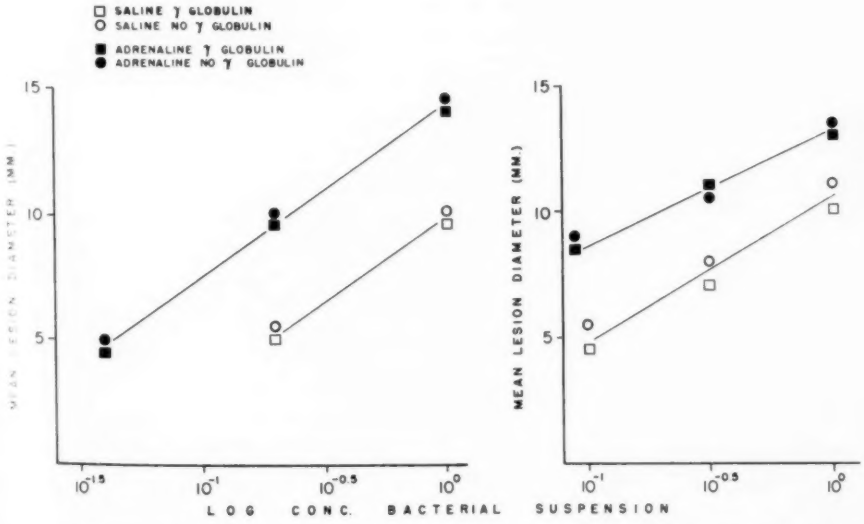

Fig. 1.—Effect of systemic administration of gamma globulin on staphylococcal lesions in the skin of guinea pigs.

development of the skin lesions, it might perhaps have an effect by enhancing the action of an antibiotic. Indeed, in many of the acute lethal experiments on mice in which benefit from gamma globulin has been reported, such benefit has been shown as a synergic effect with an antibiotic rather than as an effect of gamma globulin alone.

The possibility of a combined effect of gamma globulin and an antimicrobial drug on the local lesion was tested by finding out if the period during which the antimicrobial drug was able to influence the lesion was affected by the systemic administration of gamma globulin. Miles and his colleagues have shown that penicillin and streptomycin can reduce the size of the skin lesion produced by a number of bacteria only if they are given within 3 to 5 hours after the injection of the bacterial suspension. This corresponds with the difficulty encountered in clinical practice in affecting local foci of infection once they have become established. Anything which could prolong this decisive period during which antimicrobial drugs are able to affect the ultimate severity

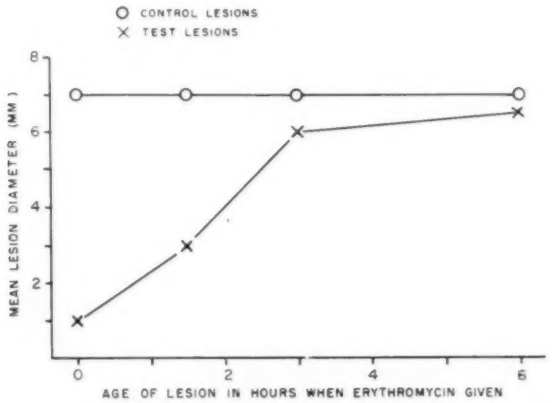

Fig. 2.—The "decisive period" of erythromycin action on local staphylococcal lesions.

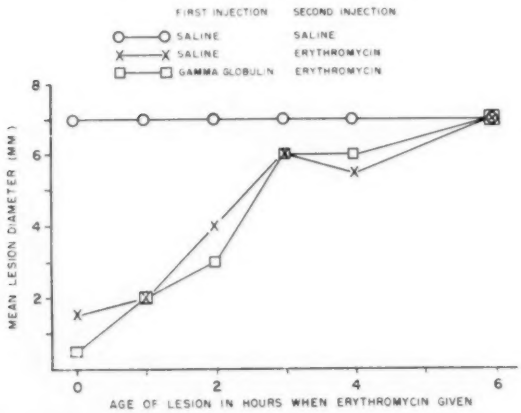

Fig. 3.—Effect of systemic administration of gamma globulin on the decisive period of erythromycin action.

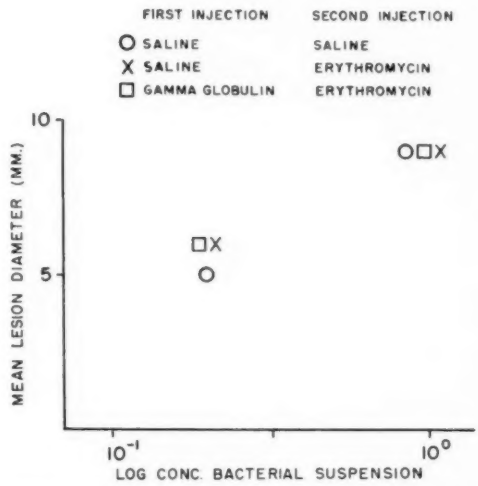

Fig. 4.—Effect of systemic administration of gamma globulin on lesions initiated 4 hours after injection of erythromycin.

of the lesion would be a valuable addition to treatment and it was interesting to see if gamma globulin could do this.

A saline suspension of staphylococci, strain S81, was prepared without addition of adrenaline and was injected at various skin sites in a number of guinea pigs at different intervals of time before the intramuscular injection of 5 mg. erythromycin in 0.2 ml. saline. Erythromycin was used because S81 is fully susceptible to it. Guinea pigs used as controls received exactly similar treatment except that they were given an intramuscular injection of saline instead of erythromycin. In several preliminary experiments there was a progressive reduction in size even of the lesions of the control group as a result of the development of nonspecific local immunity in the skin. It was found that this could be avoided by spacing the lesions more widely in the area of skin used for injection. A representative experiment may be seen in Fig. 2. It is evident that lesions initiated more than 3 hours before the injection of erythromycin are little different from those of the control group in their ultimate development. The viable count on the bacterial suspension at the end of these experiments showed little or no change from the initial count. Fig. 3 is representative of a group of experiments in which the effect of gamma globulin on the decisive period of erythromycin treatment was tested.

Three guinea pigs were given 0.5 ml. gamma globulin by intraperitoneal injection 3 hours before the series of intradermal infections was started; the other five guinea pigs in the experiment received saline. A saline suspension of S81 was injected intradermally at random sites in the skin at various intervals over a period of 6 hours. At the end of this time the three animals which had been given gamma globulin and three of the five which had received saline were injected intramuscularly with 5 mg. erythromycin. The remaining two guinea pigs received intramuscular saline. Fig. 3 shows that erythromycin has a

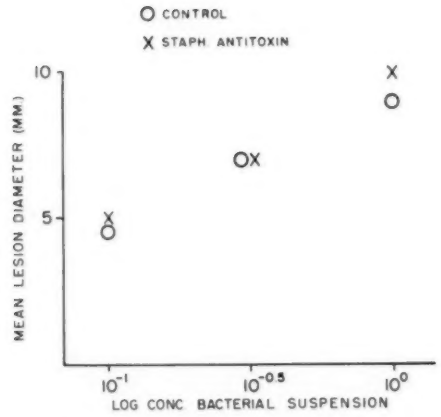

Fig. 5.—Effect of systemic administration of staphylococcal antitoxin on staphylococcal lesions in the skin of guinea pigs.

marked influence in reducing the ultimate size of the lesions if given within 2 hours of their inception; a slight effect is observed at 3 and 4 hours but lesions 6 hours old when erythromycin is given are unaffected by it. It is clear also that the decisive period is the same in the animals which have received gamma globulin as in the control animals.

Whether or not gamma globulin could prolong the decisive period in which erythromycin is able to modify the lesions was tested in another way in five further experiments, in which the erythromycin was given at a set time, either 4 or 6 hours after a series of dilutions of the bacterial suspension had been injected intradermally. By choosing this interval at the limit of effectiveness of erythromycin, it could be seen whether at any dose level the pigs receiving gamma globulin had an advantage over the control group. In one of these experiments an appreciable effect of erythromycin was still present whereas in the others its effect was no longer apparent, but in none of them did the pigs which received gamma globulin have any advantage over the control group. Fig. 4 illustrates one of this group of experiments.

Effect of Staphylococcal Antitoxin on Skin Lesions.—The reported effects of gamma globulin on acute lethal infections with staphylococci in mice might perhaps be caused by staphylococcal antitoxin contained in it. It was therefore interesting to know whether staphylococcal alpha toxin played an important role in the production of the skin lesions, since these lesions could not be modified by the systemic administration of gamma globulin. The cocci are suspended in saline and are not grown under the special conditions favorable for toxin production so that no appreciable amount of free toxin would be present when the infection is initiated. It was shown in a group of experiments, one of which is illustrated in Fig. 5, that intraperitoneal injection of 1,000 units of staphylococcal antitoxin 3 to 4 hours before the intradermal injections produced no change in the local lesions. In these experiments the control group also received horse serum by intraperitoneal injection in the form of diphtheria antitoxin. That staphylococcal antitoxin given in this way was capable of

reaching the skin in effective concentration was shown by the fact that the same dose given by the same route 4 hours before the intradermal injection of serial dilutions of staphylococcal toxin reduced its dermonecrotic titer by four- to eightfold.

Alpha Antitoxin Content of Gamma Globulin.—In considering the possibility that gamma globulin, when effective in staphylococcal infections, might be acting by virtue of its content of alpha antitoxin, it was necessary to find out how much antitoxin is present in the gamma globulin preparation. This was done in two ways. The lethal dose of a preparation of alpha toxin from staphylococci, strain Wood 46, was first determined. The standard preparation of staphylococcal antitoxin was then titrated in parallel with the gamma globulin preparation, in the presence of a lethal dose of toxin in each tube and using as the end point partial hemolysis of washed 2 per cent rabbit red cells. This procedure was done on three different batches of gamma globulin. The content of antitoxin, which was similar in the three lots, was 12 to 15 units per milliliter. The staphylococcal antitoxin content of gamma globulin was also determined by mixing varying amounts of standard antitoxin or of gamma globulin with a standard lethal dose of toxin, and administering these mixtures to mice in 0.5 ml. volumes by intravenous injection. Table I shows such an experiment. Administration of 0.0125 ml. gamma globulin neutralized the lethal effect of staphylococcal toxin as effectively as 0.25 unit of standard antitoxin, so that by this estimation the gamma globulin contained 20 units of antitoxin per milliliter.

Experiments in Mice.—As the work on guinea pigs had shown that gamma globulin was ineffective in modifying a local form of staphylococcal infection and that the development of this focus was also unaffected by antitoxin, further experiments were done using an acute lethal infection on mice in which humoral factors might perhaps show a more significant protective effect. More specifically, an attempt was made to find out if any protective action of gamma globulin in acute infections was due to its content of alpha antitoxin or whether some other factor such as specific antibacterial antibody would have to be invoked. In a number of tests in which mice were inoculated intraperitoneally with suspensions of *Staph. aureus* "Smith" in 5 per cent gastric mucin it was possible to show that previous injection of antitoxin gave partial protection but

TABLE I. ESTIMATION OF ALPHA ANTITOXIN CONTENT OF GAMMA GLOBULIN BY ITS EFFECT IN NEUTRALIZING INTRAVENOUS ALPHA TOXIN IN MICE

DOSE OF STAPHYLOCOCCAL ANTITOXIN PER MOUSE (UNITS)	DEATHS IN 48 HOURS IN GROUPS OF 5 MICE	DOSE OF GAMMA GLOBULIN PER MOUSE (ML.)	DEATHS IN 48 HOURS IN GROUPS OF 5 MICE
0.5	0	0.1	0
0.25	0	0.05	0
0.125	5	0.25	0
0.062	5	0.0125	1
0.031	5	0.006	5
0.015	5	0.003	5
		0	5

TABLE II. EFFECT OF STAPHYLOCOCCAL ANTITOXIN AND OF GAMMA GLOBULIN ON MORTALITY CAUSED BY INTRAPERITONEAL INJECTION OF STAPH. AUREUS "SMITH" IN 5 PER CENT GASTRIC MUCIN

PRELIMINARY INJECTION	DEATHS IN GROUPS OF 10 MICE AT	
	24 HOURS	7 DAYS
Staph. antitoxin 200 units/mouse	2	9
Staph. antitoxin 50 units/mouse	4	10
Staph. antitoxin 10 units/mouse	8	9
Gamma globulin 0.2 ml./mouse	8	8
Gamma globulin 0.05 ml./mouse	10	10
Diphtheria antitoxin 200 units/mouse	10	10

that gamma globulin did not. The protection afforded by antitoxin was not absolute, but merely delayed the death rate on the first and second days compared with the control group. Thus, in the experiment shown in Table II 50 units and 200 units of antitoxin gave appreciable protection for the first 24 hours, but 10 units of antitoxin, 0.2 ml. and 0.05 ml. gamma globulin had no effect. A large dose of antitoxin is needed to demonstrate any effect on this type of infection. If the claimed protective action of gamma globulin is due to its antitoxin content, such a protective action would not be demonstrable in this system, since the enormous quantity of 2.5 ml. of undiluted gamma globulin would have to be injected into each mouse in order to provide 50 units of staphylococcal antitoxin.

The failure to demonstrate protection by gamma globulin in this type of infection was also shown in experiments in which different amounts of chloramphenicol, with or without 0.05 ml. gamma globulin, were injected into mice immediately before the injection of staphylococci in mucin. This titration of the protective effect of chloramphenicol gave the same results in the mice which received gamma globulin as in those which did not. An experiment of this type is illustrated in Table III.

DISCUSSION

Staphylococcal infections in man are most commonly intractable to treatment when sizeable local foci of infection have become established at sites inaccessible to surgical drainage, as on a heart valve. Even more superficially

TABLE III. EFFECT OF VARIOUS DOSES OF CHLORAMPHENICOL WITH AND WITHOUT GAMMA GLOBULIN ON MORTALITY FROM INTRAPERITONEAL INJECTION OF STAPH. AUREUS "SMITH" IN MUCIN

DOSE OF CHLORAMPHENICOL (MG. PER KG.)	DOSE OF GAMMA GLOBULIN (ML.)	DEATHS IN GROUPS OF 10 MICE IN 7 DAYS
250	0.05	1
50	0.05	1
10	0.05	6
2	0.05	8
0	0.05	9
250	0	1
50	0	2
10	0	6
2	0	5
0	0	7

situated local foci of infection are often difficult to heal as are, for example, some multiple abscesses of the breast and chronic staphylococcal skin infections. It is important therefore to ask of any proposed protective agent whether it can diminish the likelihood that a local abscess will become established. The experiments on guinea pig skin show that gamma globulin does not affect the development of this type of local lesion and cannot prolong the period during which an antibiotic is able to affect the fate of the lesion.

It still remains to ask whether gamma globulin can exert any action on more generalized staphylococcal infections, and whether, if such an effect is shown, it is attributable to specific antitoxin contained in the globulin preparation. If the effect of gamma globulin is simply a function of its contained antitoxin, the antitoxin would be more easily given using a specific preparation which contained 2,000 units per milliliter than by using gamma globulin which contained 10 to 20 units per milliliter. Any possible role of gamma globulin in treatment would be very limited in its scope since it is clear that although antitoxin can prevent death from staphylococcal toxin and from massive systemic staphylococcal infections, it can neither prevent nor affect the course of the bacteremia and suppurative complications of these infections.[9-11] The present experiments do not answer this question in any positive way, but in the acute peritoneal infection in mice which was used no protection by gamma globulin was demonstrable, and the dose of antitoxin necessary to show protection in this system could not be achieved in the form of gamma globulin in any practicable injection volume. Antitoxin has not been compared with gamma globulin in previous published work on peritoneal infections in mice but it does seem possible that the particular system used was one in which staphylococcal toxin played a more important role than in ours, allowing the demonstration of some protective effect even with the small amount of antitoxin contained in gamma globulin. The work on intracerebral staphylococcal infections in mice gives strong support for this idea, since Sonea and his colleagues[5] have studied the effect of both preparations, albeit in separate groups of experiments. Protection by gamma globulin could be demonstrated only when doses of the order of 0.2 to 1.0 ml. were used, which, by our estimations, would contain about 20 units of antitoxin. In experiments on antitoxin in which the same challenge was used,[5] marked protection could be shown by 0.5 ml. of 1:100 antitoxin, or about 10 units, and slight protection by 1 unit.[12] This does give a close correspondence between the two protective agents in terms of the antitoxin content.

Fisher and Manning[7] feel that the protective effect of gamma globulin which they have demonstrated in a number of acute infections in mice is due to its content of specific antibacterial antibodies because they found that absorption of gamma globulin with a suspension of *Staph. aureus* specifically removed its activity against this organism. The effect of this procedure on the antitoxin titer of the preparation was not studied. Certainly previous work on experimental staphylococcal infection has signally failed to show protection by specific antibacterial antibody, and this work has been concerned with high titer antibodies produced in response to specific vaccination procedures.

Forssman[13] claimed that antibacterial as opposed to antitoxin immunity was demonstrable, but other workers have either failed to show that it exists[14] or, if they have induced antibacterial immunity, have shown that it is nonspecific.[15]

SUMMARY

Systemic administration of gamma globulin does not affect the course of a local staphylococcal lesion in the skin of guinea pigs and does not prolong the period of time during which an antibiotic is able to affect the course of such a lesion.

No protective effect of gamma globulin is demonstrable in an acute staphylococcal infection in mice of the type described here. Staphylococcal antitoxin does have a protective effect in this type of infection but the amount needed to show this effect could not be provided in the form of gamma globulin in any reasonable volume for injection.

Gamma globulin contains 10 to 20 units of staphylococcal antitoxin per milliliter.

It is suggested that the reported protective effects of gamma globulin in experimental staphylococcal infections may be attributable to its content of staphylococcal alpha antitoxin, and that gamma globulin has nothing to offer in the prevention or treatment of focal staphylococcal lesions.

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THROMBOCYTOPENIA AFTER INTRAVENOUS INJECTION OF INDIA INK

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By means of isotope-labeled platelets transfused into recipient animals (rabbits) it has been possible to demonstrate that the brief thrombocytopenia which occurs after injection of India ink is due to temporary sequestration of the platelets. The platelets loaded with particles of India ink are detained, perhaps, in organs of the reticuloendothelial system and thereafter they gradually return to the circulating blood. Platelets seem to play a role in the defense of the organism to the invasion by foreign particles into the blood.

IN 1926 Tait and Elvidge[1] described experiments in rabbits in which the platelets disappeared from the blood immediately after injections of quartz particles, barium sulfate, carmine, or India ink. They made an extensive study using quartz powder and found that the platelet count recovered only gradually, requiring about 6 days to return to the original level. We have repeated this experiment using India ink instead of quartz. The platelets disappeared promptly, as reported by Tait and Elvidge, but recovery of the platelet count was a matter of hours rather than days. The difference between the reaction to quartz and the reaction to carbon (India ink) suggested that the fate of the platelets was not the same in the two experiments. Using platelets with an isotopic label so that it was possible to trace them after injection of India ink, we have tried to elucidate the mechanism of the thrombocytopenia and recovery.

MATERIALS AND METHODS

All syringes and other glassware were coated with silicone, and EDTA was used as anticoagulant. Platelets were counted by the direct method,[2] using contrast microscopy.[3] India ink used for the experiments was Guenter-Wagner brand (Pelikan waterproof drawing ink, black). There were 130 to 150 mg. of dry solids per milliliter of ink. The ink was prepared for injection by filtering, autoclaving for ten minutes, and diluting with 10 per cent dextrose to make a 2 per cent (v/v) solution (25 to 30 mg. per milliliter). The usual dose was 15 to 20 ml. of this solution injected into the rabbit's ear vein in 1 to 2 minutes.

The experiments were performed with adult rabbits of either sex weighing 2.5 to 3.5 kilograms. Donor animals of the radioactive, labeled platelets received a single intravenous injection of 350 μc of P^{32} according to the procedure of Adelson and co-workers.[4] After 5 or 6 days, 30 to 40 ml. of whole blood was obtained by cardiac puncture and transfused immediately into an ear vein of a recipient rabbit, prepared by a phlebotomy of 30 to 40 ml.

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Because there is an immediate, temporary sequestration of transfused platelets,[4] the first samples were not taken until 2 or 3 hours after the transfusion. Thereafter, samples were taken at 24-hour intervals. The platelets were separated by differential centrifugation and washed repeatedly to free them of plasma. In the final suspension they were counted microscopically and a measured volume was transferred to the copper planchet and dried, and the radioactivity was counted by means of a Geiger tube with a thin window. To avoid the problem of physical decay all radioactive samples from each experiment were counted at the same time. From these values it was possible to compute the specific activity of the platelets in terms of counts per platelet per unit of time. The radioactivity of the platelets of the recipient rabbits was determined as least once before the administration of India ink and several times afterward.

In some experiments the blood from one donor animal was transfused into two recipients at the same time and the fate of the radioactive platelets was studied in both. One received India ink, whereas the other did not.

In other experiments the donor animal was given P^{32} and after 5 days was given an injection of India ink. When the platelet count had returned to normal (20 hours later) blood was taken and transfused into a recipient rabbit. The specific activity of the transfused labeled platelets was measured each day for several days.

RESULTS

The preliminary platelet counts of the rabbits used in these experiments varied from 138,000 to 750,000 per cubic millimeter with a mean of 416,000. When the animals were given 2 or 3 ml. of the 2 per cent solution of India ink, the platelet counts showed little response. When 15 to 20 ml. of the solution was given (400 to 500 mg. of ink), however, there was an abrupt drop in the platelet count to levels of 1,000 to 12,000 per cubic millimeter. Thereafter, the count gradually recovered and within 2 to 4 hours it had returned to the initial level. In Fig. 1 the results of a typical experiment are shown.

The results of the experiments with radioactive platelets are shown in Fig. 2. In a typical experiment, the rabbit was given an injection of India ink 4 hours

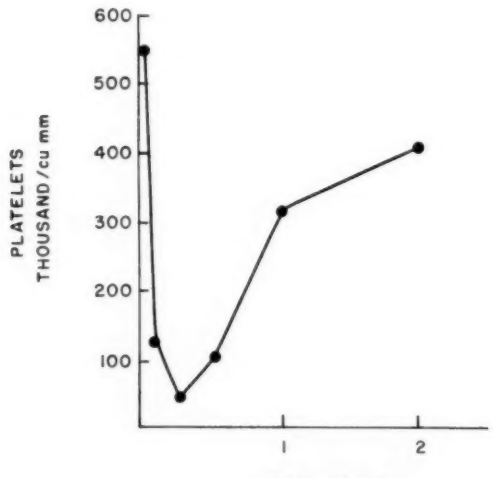

Fig. 1.—Thrombocytopenia induced in a rabbit by injection of 15 ml. of a 2 per cent solution of India ink at time zero.

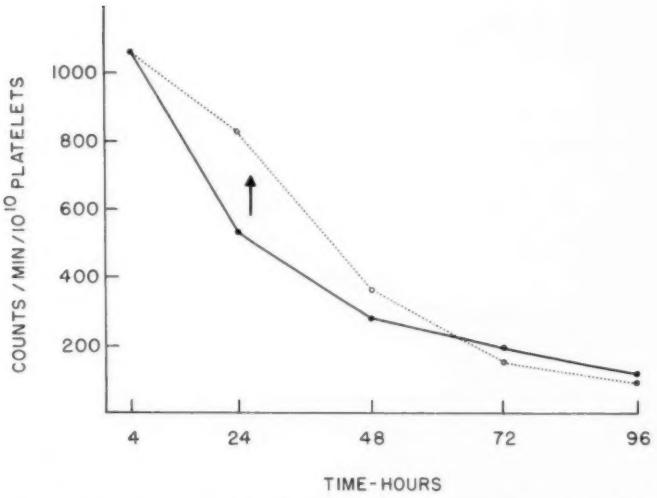

Fig. 2.—The effect of injection of India ink on the radioactivity of transfused P^{32}-labeled platelets. The solid line represents the loss of radioactive platelets in a series of control animals. Each point is the average of at least eight experiments. The broken line represents the loss of platelets in animals treated with India ink at the time indicated by the arrow. Each point is the average of seven experiments. After the injection of ink, the platelet count fell almost to zero and then recovered.

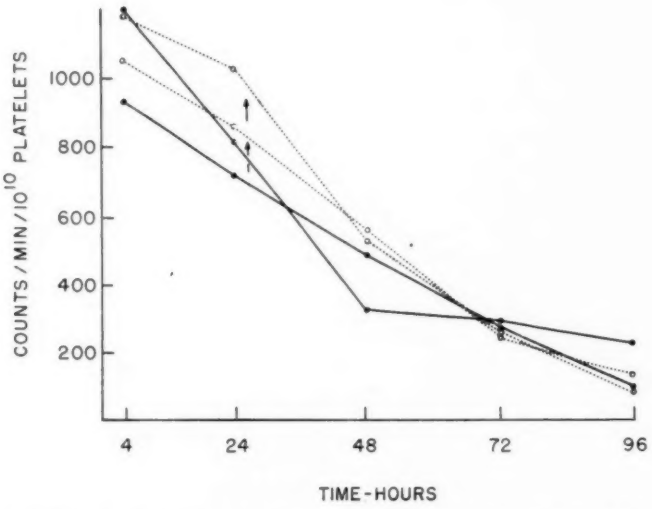

Fig. 3.—The effect of an injection of India ink on the rate of loss of transfused radioactive platelets. Two experiments in which the recipient animals received labeled blood at the same time from the same donor. See Fig. 2 for explanation.

after transfusion of P^{32}-labeled platelets. Within 3 minutes the platelet count fell from the posttranfusion value of 350,000 per cubic millimeter to 6,000. It recovered promptly so that 18 hours after the injection of ink the platelet count had returned to 336,000 per cubic millimeter. The specific activity of the platelets before injection of ink was 2,400 counts per minute per 10^{10} platelets. The next day, after the platelet count had recovered from the ink

induced thrombocytopenia, the specific activity was 1,200 counts per minute per 10^{10} platelets. This decrease of specific activity in 24 hours was of the same order of magnitude as that observed in control animals given radioactive platelets but no injections of India ink.

A single rabbit served as donor for two recipients in four experiments with radioactive platelets. When one of each pair was injected with ink, the platelet count fell and recovered but the radioactivity of the platelets was not influenced by the episode of thrombocytopenia (Fig. 3).

When a donor animal was given an injection of India ink 20 hours before his blood was drawn and transfused into an untreated recipient, only a small proportion of the transfused radioactive platelets could be found in the recipient's blood (Fig. 4).

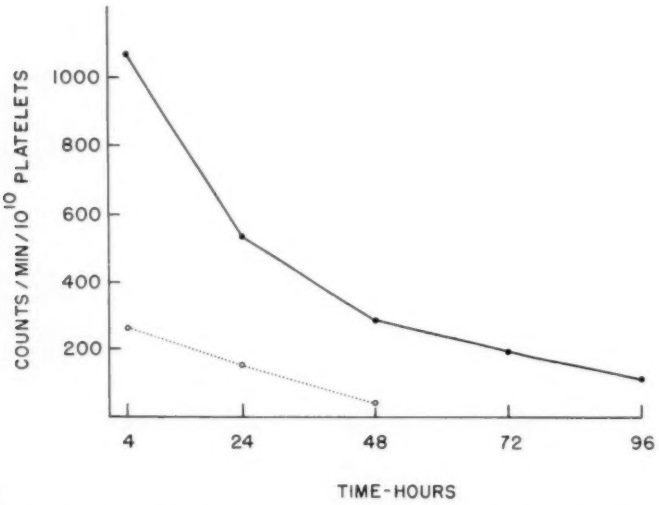

Fig. 4.—The solid line is the group of control animals shown in Fig. 2. The broken line presents the average values of three experiments in which the *donor* animal was treated with India ink 24 hours before its blood was drawn and transfused into a nomal recipient. After the injection of ink, the platelet count fell almost to zero and recovered. Thus platelets which had been sequestered were transfused but few of them survived the manipulation.

After the injection of India ink the plasma of the injected rabbit was found to be pigmented by the carbon particles, and it was impossible to separate this pigment from the platelets either by sedimentation, differential centrifugation, or washing. Microscopy demonstrated that each platelet was frosted with carbon. Observations on blood smears made some hours after India ink injection showed groups of agglutinated platelets covered with India ink particles, and the histologic preparations of lymph node, liver, bone marrow, and spleen showed agglutinated India ink particles with huge numbers of platelets.

DISCUSSION

The isotope experiments indicate that the disappearance of platelets from the circulating blood after the administration of India ink was not due to

destruction. The gradual recovery of the platelet count was not accomplished by the release into the circulation of a new generation of platelets. If the recovery of the platelet count after India ink were due to a new crop of platelets, we would have been unable to measure any radioactivity in these new platelets. There is no significant reutilization of P^{32} from destroyed platelets.[4] We think, therefore, that the platelets, loaded with India ink particles, underwent a process of sequestration.

The loading of platelets with foreign particles is a well-established fact: during acute infectious diseases in man and after experimental inoculation of animals with microorganisms, the number of platelets decreases.[5] The introduction of bacteria as well as inert colloidal material will produce clumping of the platelets in birds and rabbits.[6, 7] The adhesion of the platelets and the bacteria seems, however, a surface phenomenon,[8, 9] and platelet loading is believed essentially similar to the first phase of phagocytosis—a passive process depending on the modification of the microorganism and of the platelet by the surrounding medium.[10] Our histologic observations, together with our radioactive studies, have led us to think that a similar mechanism may be at the basis of the disappearance of the platelets after injection of massive dosages of India ink. The platelets, loaded with India ink particles, were agglutinated and went into hiding in the reticuloendothelial system. Subsequently the platelets were again released. The behavior of the platelets in this manner may be functional, helping to clear the blood of the injected foreign particles.

It is probable that in the course of the loading with India ink the platelets underwent some functional damage. When prelabeled blood of donor animals which had received injections of India ink was transfused into recipient animals 17 to 20 hours after the administration of ink, only a small proportion of the radioactive platelets could be recovered from the recipient animals, which suggested that the transfused platelets had been damaged by the ink and had been removed and destroyed by the reticuloendothelial cells of the recipient animal.

SUMMARY

By means of isotope-labeled platelets transfused into recipient animals it has been possible to demonstrate that the thrombocytopenia after injection of India ink was due to the temporary sequestration of platelets. The platelets loaded with India ink particles were detained in the reticuloendothelial system and were thereafter gradually returned to the circulating blood. Platelets seem to play an important role in the defense mechanism of the animal organism to the invasion of foreign particles.

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MEASUREMENT OF GASTROINTESTINAL BLOOD LOSS IN SUBJECTS TAKING ASPIRIN

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Previous authors have suggested the frequency of occult gastrointestinal bleeding in subjects taking aspirin. In this study the extent of the blood loss is investigated by means of a radioactive chromium^{51} *method in which patients and volunteers are used as their own control. Sixty-nine per cent of subjects were considered to be losing blood while they were taking 40 grains of aspirin in divided doses daily, and 17 per cent lost more than 6 ml. (an average twentyfold increase over control levels). The loss of blood was not related to the sex or age of the subjects, hematologic changes, salicylate level, or gastric symptoms. A discussion of possible mechanisms and effects is included.*

THE occurrence of *massive* gastrointestinal hemorrhage after salicylate ingestion has been stressed recently by several authors,[1-4] although the incidence varies widely in different series. Perhaps the extensive use of these drugs has obscured the importance of such observations. The possibility that salicylates may cause occult bleeding in a majority of subjects was suggested by the work of Lange,[5] who noted that the presence of benzidine-positive stools was 2½ times as great in patients taking aspirin, as in the same patients when the drug was withdrawn. Stubbe,[6] in a well-controlled series, found positive benzidine tests on stools from over 70 per cent of patients and volunteers on moderate aspirin ingestion, and this observation was confirmed by Alvarez and Summerskill.[2]

The aim of the present study was to confirm these results and to make a quantitative assessment of the occult blood loss. For this purpose, the use of radioactive sodium chromate to tag red cells and the subsequent measurement of radioactivity in the stools as described by Owen and co-workers,[7] Bannerman,[8] and Ebaugh and associates[9] appeared to be the simplest and most accurate method available. Colorimetric methods of measuring blood in the stools, such as that used by Andrews and Oliver-González[10] are laborious and a spectrophotometric method recently described[11] is admitted by the authors to be less exact.

PROCEDURE

Experimental Plan.—Twenty-five patients who were expected to remain in the hospital for at least 16 days were selected from the wards. The patients studied were under treatment

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for a variety of illnesses (see Table I), but any subject with present or past known gastrointestinal lesions was excluded. Roentgenographic examinations were performed on only six subjects. Ten healthy volunteers investigated consisted of personnel in the same hospital. There were nineteen women and sixteen men in this series.

The subjects were placed on a modified meat-poor diet and were allowed only well-cooked poultry and occasionally veal as meat. All salicylate therapy was discontinued and stool collections were not performed on women during the menstrual period. Before any patient was admitted to the study, at least four consecutive daily stool specimens were tested and all had to give a negative result with the benzidine test. In eight patients, a faint positive reaction was obtained in the first two stools, which reverted to negative by the third day of the meat-poor diet.

After this control period, the patients' red cells were labeled with radioactive chromium^{51} and on the same day salicylates were started. On each day of salicylate therapy, two tablets of aspirin (U. S. P.), 5 gr. each (330 mg.),* were crushed and swallowed with water 4 times daily on an empty stomach within half an hour before meals, a total of 40 gr. daily. Stool specimens were collected daily and a sample was tested for occult blood. After 4 to 6 days, a 4 day fecal collection was made while the subjects continued to take aspirin. On the third day of the collection period blood was drawn for the measurement of radioactivity, prothrombin time, salicylate level, and platelet count. A Rumpel-Leeds test for capillary fragility was performed in some subjects and was consistently negative. At the end of the stool collection, the tablets were stopped and the subjects were asked to describe any symptoms which occurred while they were taking aspirin with particular reference to the presence of gastrointestinal distress. After at least 6 days without salicylates, when no occult blood was detectable in the stools, a 3 day control stool collection was made on over 50 per cent of the patients, and the radioactivity in stool and blood was again measured. In eleven subjects this control stool collection was obtained before the salicylates were administered but the conditions of the study were otherwise unchanged. Five studies on four patients with rheumatic disorders, who were taking over 40 gr. of aspirin daily, were performed and one patient under treatment with anticoagulants (acenocoumarin) for thrombophlebitis was examined when given 40 gr. daily.

METHODS

1. *Benzidine Test.*—The modified Gregerson test described by Needham and Simpson[12] was used on stool specimens before homogenization: the previously prepared benzidine and barium peroxide powder was suspended in glacial acetic acid shortly before use. The final dilution of benzidine was 0.5 per cent. The reaction was considered 3 plus positive with the immediate development of the blue color, 2 plus when the color appeared within 15 seconds, 1 plus in 15 to 30 seconds, and negative if the color developed after 30 seconds.

2. *Collection and Preparation of Fecal Specimens.*—The 4 day stool collections were passed into a weighed 2 quart Mason jar directly or transferred from a bed pan and the jar was reweighed to estimate stool weight. Approximately 1 to 1.5 parts of water were added to 1 part of feces depending on stool consistency and the stools were homogenized in the jar by means of a blender† to obtain a thick suspension. Two milliliter aliquots of this suspension were placed into 10 ml. celluloid centrifuge tubes and the radioactivity emitted was measured in a well-type scintillation counter.

3. *Labeling of Red Cells With Radioactive Sodium Chromate.*—Twenty to 30 ml. of whole blood was drawn and placed into a sterile 75 ml. tag vial which contained 1 ml. of acid citrate dextrose solution‡ per 4 ml. of whole blood. $Na_2Cr^{51}O_4$, 140 to 200 μc, was added and mixed for 30 minutes using an Eberlach variable rotator, at which time 100 mg.

*For the sake of simplicity, the dose of aspirin has been expressed in grains.

†Multimixer manufactured by Lourdes Instrument Company, Brooklyn, N. Y.

‡Pyrogen-free acid citrate dextrose solution for tagging red blood cells with Chromitope sodium, E. R. Squibb & Sons, New York, N. Y.

of ascorbic acid was added and the solution was again mixed gently for 15 minutes. The red cells were not washed prior to reinjection into the patient because over 80 per cent of the chromium becomes bound to red cells when this method is used.

4. *Radioactive Counting.*—The chromium^{51} in the specimens was assayed in a well-type scintillation counter and the physical decay of the radioactive chromium was measured with a standard. All measurements were corrected for background activity. Immediately after completion of the collection period, 1 ml. specimens of whole blood were counted for 100 seconds and the stool aliquots for 15 minutes to give a counting error of less than 2.5 per cent. The number of milliliters of whole blood in the stools per day was calculated by the following formula: where c.p.s. = counts per second, milliliters of blood lost per day =

$$\frac{\text{c.p.s. per gram of homogenized stool} \times \text{weight of homogenized stool}}{\text{c.p.s. in 1 ml. of circulating blood} \times \text{days of stool collection}}$$

Blood loss of under 0.2 ml. was reported as "less than 0.2 ml."

5. *Platelet Counts.*—Platelet counts were performed according to the method of Rees and Ecker[13] modified by the use of 3.8 per cent sodium chromate alone.

6. *Prothrombin Times.*—Prothrombin times were measured using the Link-Shapiro modification of Quick's one stage prothrombin time method.[14]

7. *Blood Salicylates.*—Blood salicylates were estimated by means of the method of Brodie and co-workers.[15]

RESULTS

Table I gives the daily loss of blood in the stool as measured by the loss of radioactivity. It should be emphasized that although the results are given as milliliters of whole blood lost in the stools per day, this represents the appearance of radioactivity and there is no evidence that all the chromium in the stool represents red cells and not in part excretion of chromium into the gut released from circulating tagged red cells. However, the increase in radioactivity in the stools of patients who were taking aspirin is so striking that this component could only have affected the figures to a minor degree. The quantitative study of gastrointestinal bleeding by the measurement of chromium^{51} activity in the stool is dependent on the fact that after intravenous injection of patients' labeled red cells insignificant amounts appear in the stool in the absence of bleeding; if these red cells are placed in the bowel the chromium is not reabsorbed.[7, 9] This suggests that the figures for radioactivity in the stools represent a true estimate of loss of blood into the bowel.

The benzidine test converted from negative to positive by the third or fourth day of aspirin ingestion in most subjects, and a comparison between the results of the benzidine test as employed (with the patients on a modified meat-poor diet) and the number of milliliters of blood lost per day as measured by the radioactive chromium method showed that a negative reaction indicated bleeding of less than 1.7 ml. per day in all but one specimen. No clear correlation between the degree of bleeding and the intensity of the benzidine test was found. Six of 23 stool specimens showed a 1 plus reaction with a blood loss of less than 1.7 ml. per day; the average was 2.7 ml. with a range of 0.7 to 8 ml. per day. A 2 plus reaction occurred in 10 stools with an average of 5.1 ml. and a range of 2.0 to 8.1 ml. per day, and a 3 plus reaction was obtained

TABLE I

PATIENT	AGE (YR.)	SEX	DIAGNOSIS	ML. OF WHOLE BLOOD BLOOD LOST DAILY			PROTHROMBIN TIME (%)	COMMENTS
				CONTROL PERIOD	ASPIRIN PERIOD	DIFFERENCE		
Patients Taking 40 gr. of Aspirin Daily.—								
H. B.	64	F	Arteriosclerotic heart disease	< 0.2	3.5	3.3	60	
R. S.	28	M	Pneumonia		6.5		75	
M. L.	44	M	Hypertension		3.0		100	
A. M.	67	F	Fractured spine	< 0.2	2.5	2.3	100	
M. T.	63	F	Hallux valgus		0.2		100	
J. D.	53	F	Hallux valgus		2.8		100	
M. L.	74	F	Fractured spine	0.2	1.4	1.2	100	
J. P.	35	F	Accoustic neuroma		1.2		100	
R. W.	43	F	Myxedema		2.4		100	
M. H.	64	F	Fractured spine	1.9	2.4	0.5	100	
W. R.	22	M	Infectious hepatitis	< 0.2	1.5	1.3	100	
M. M.	31	M	Cellulitis		7.9		60	
J. Pr.	64	M	Cellulitis	< 0.2	2.8	2.6	60	
A. S.	56	M	Cellulitis		0.7		100	2 day stool collection
P. P.	70	M	Benign prostatic hypertrophy		2.0		100	
J. W.	53	M	Cor pulmonale	0.5	1.1	0.6	100	
R. H.	18	M	Sickle cell disease		2.1		100	
R. S.	28	M	Pneumonia		0.5		51	
J. L.	47	M	Knee fusion	0.8	1.8	1.0	100	
E. D.	73	M	Contact dermatitis		2.8		100	
I. R.	51	F	Diabetes	0.7	1.1	0.4	100	
J. D.	49	F	Infectious hepatitis	< 0.2	0.3	0.1	61	
P. E.	37	M	Cor Pulmonale	1.7	3.6	1.9	100	
S. E.	65	F	Fractured spine		1.3		100	
C. J.	35	F	Infectious hepatitis		1.3		42	
M. N.	61	F	Lung carcinoma		3.3		25	On anticoagulation therapy
Volunteers Taking 40 gr. of Aspirin Daily.—								
B. S.	37	M	Normal	0.6	2.9	2.3	100	
D. K.	26	F	Normal	< 0.2	3.3	3.1	100	
V. C.	23	F	Normal	0.2	2.6	2.4	100	
E. L.	24	F	Normal	1.0	13.8	12.8	100	
E. W.	26	M	Normal	0.8	3.0	2.2	100	
C. W.	24	F	Normal	< 0.2	8.1	7.9	63	
A. A.	23	F	Normal	< 0.2	4.7	4.5	100	
E. H.	22	F	Normal	0.5	9.2	8.7	100	
D. P.	24	F	Normal	0.2	2.8	2.6	75	
R. P.	29	M	Normal	0.2	6.8	6.6	100	
Patients Taking More Than 40 gr. of Aspirin Daily.—								
N. R.	24	F	Rheumatic fever		4.9		60	Aspirin, 100 gr.
N. R.	24	F	Rheumatic fever		5.4		60	Aspirin, 60 gr.
H. B.	56	F	Rheumatoid arthritis		7.3		66	Aspirin, 60 gr.
E. R.	21	M	Rheumatic fever	< 0.2	4.3	4.1	64	Aspirin, 120 gr., prednisone, 40 mg.
E. B.	18	F	Rheumatic fever		3.0		100	Aspirin, 90 gr., prednisone, 30 mg.

with a blood loss as low as 4.3 ml. per day in one specimen. No closer correlation was noted when the benzidine reaction was compared to the daily blood loss per 100 grams of wet stool weight.

Control periods without aspirin were available on 21 subjects. The average loss of whole blood per day was 0.5 ml. with a standard deviation (S. D.) of 0.5 and a range of 0.2 to 1.9 ml. One patient bled more than 1.7 ml. per day without any obvious pathologic cause. These figures are lower than those quoted by Roche and coauthors[16] and Ebaugh and associates[9] but agree with those of Hughes-Jones[17] and Pierson and co-workers.[18] The latter have studied forty-one subjects who were not bleeding and found an average loss of 0.5 ml. per day. Although patients were selected after four benzidine-negative stools, only three subjects were refused because of the appearance of positive stools without an obvious pathologic condition of the gastrointestinal tract, whereas twenty-four were lost to the study for other reasons. It therefore seems reasonable to state that the average blood loss per day is below 1.0 ml. and that the upper limits of normal can be set below 1.7 ml.

Thirty-five subjects were studied while taking 40 gr. of aspirin daily. Eleven (31 per cent) bled less than 1.7 ml. per day and were considered to be "not bleeding" (Group I). However, each of the five subjects in this group on whom an isotopic study was made when not taking aspirin showed an increase in daily blood loss while on the drug which averaged 0.7 ml. (almost a three-fold increase). Sixty-nine per cent bled more than 1.8 ml. per day and they have been arbitrarily divided into two groups: those bleeding up to 6 ml. per day (Group II), and those bleeding more than 6 ml. per day (Group III). Eighteen subjects (52 per cent of the total) who appear in Group II bled an average of 2.8 ml. per day and six (17 per cent) in Group III bled an average of 8.8 ml. per day. All the ten volunteers bled, an average of 5.7 ml. per day, four appearing in Group III, whereas the fourteen of twenty-five *patients* who bled averaged only 3.3 ml. per day (S. D. = 1.66); only two lost more than 6.0 ml. per day. The difference is not statistically significant. A volunteer (E. L.) showed the greatest bleeding, 55 ml. in the 4 day study period with a control loss of 1 ml. per day. Five studies on the patients with rheumatic diseases who were taking more than 40 gr. of aspirin daily (range 60 to 120 gr., two patients also taking prednisone) showed that all bled, with an average of 5.0 ml. per day, whereas the patient receiving anticoagulants lost 3.3 ml. per day when taking 40 gr. of aspirin daily.

The average age of the group of volunteers was lower than that of the patients studied, and since these all bled, there is an apparent increased incidence in bleeding in the younger subjects. Among the twenty-five patients, there was no correlation between age and the incidence or magnitude of bleeding. No significant difference was shown between males and females.

Hematologic.—The platelet count was abnormal in only one patient with a level of 68,000. He showed no bleeding while he was taking aspirin.

The Rumpel-Leeds test was negative in all the subjects in whom it was performed.

Prothrombin times were abnormal in six patients, ranging from 42 to 75 per cent, with an average of 58 per cent. Three were in Group I, including C. J. who showed the lowest figure, 42 per cent. The result in two volunteers was slightly

abnormal, 63 and 75 per cent, and both bled. Three of four patients who were receiving larger doses of aspirin had abnormal prothrombin times of 60 to 65 per cent.

Serum salicylate levels drawn 10 to 12 hours after the evening dose of aspirin ranged from "none detectable" to 17.0 mg. per 100 ml. with an average of 4 mg. per 100 ml. The salicylate level showed no correlation with the presence of bleeding or with gastric distress.

Gastric Distress.—Six of the twenty-five patients described some gastric distress while taking aspirin: in only one was it considered severe (C. D.). However, the study had to be discontinued in two other patients because of excessive gastric distress. Seven of ten volunteers complained of symptoms, severe in two and mild in five. There was no correlation with the presence of bleeding.

In Group I, two of eleven patients complained of mild distress. In Group II, three patients and four volunteers of eighteen subjects complained of distress, symptoms being severe in three. In Group III, one patient and three of the four volunteers described mild symptoms (see Table II). E. L., the volunteer who lost 55 ml. of blood in a 4 day period had no complaints. Two of four patients taking over 40 gr. of aspirin daily had mild symptoms, whereas the patient receiving anticoagulants described severe distress.

TABLE II. GASTRIC DISTRESS COMPARED TO DEGREE OF BLEEDING

GASTRIC DISTRESS	PATIENTS			VOLUNTEERS		
	GROUP I	GROUP II	GROUP III	GROUP I	GROUP II	GROUP III
Severe		1			2	
Mild	2	2	1		2	3
None	9	9	1		2	1

DISCUSSION

This investigation agrees with the results of the study by Stubbe[6] which showed that about 70 per cent of people taking aspirin by mouth in reasonable doses lose blood in the stools. The dosage of two tablets 4 times daily was administered as about equivalent to the average ingestion of patients with painful skeletomuscular diseases. Stubbe found that when the dosage was reduced to 1.5 Gm. per day no fall in the percentage of subjects who passed benzidine-positive stools resulted, when compared to those taking 3.0 Gm. daily.

Tablets of acetylsalicylic acid (U. S. P.) were used during this study and were given crushed with water to minimize the potential effect of trauma of larger particles on the pharyngeal, esophageal, and gastric mucosa. The medication was taken on an empty stomach to correspond to the practice of most patients; however, Stubbe's patients received the pills crushed with milk or water *after* meals. Placebo tablets were not used during the control periods as it did not appear reasonable to expect blood loss into the gastrointestinal tract from them.

As this investigation was planned as a short-term study, there is no information whether prolonged administration leads to either amelioration or

exacerbation of the bleeding. However, Stubbe's patients all had rheumatic diseases, had taken aspirin chronically, and were observed for over 2 to 3 weeks so that the incidence of bleeding at least did not fall during long-term therapy.

Hematologic abnormalities and physical or functional gastric changes have been suggested as the cause of the alimentary bleeding during aspirin ingestion.

Thrombocytopenia accompanying hypersensitivity to aspirin is a rare phenomenon.[19] Hypoprothrombinemia caused by the Dicumerol-like action of the drug occurs with massive dosage and the evidence has been reviewed by Smith.[20] Fricke[21] has described depression of a prothrombin conversion factor. Capillary damage, either direct[22] or associated with a local allergic phenomenon,[23] has been postulated. This series has failed to substantiate the significance of any of these hematologic disturbances in occult bleeding.

Neither changes in gastric acidity nor volume[24] have been confirmed[25, 26] after salicylate ingestion, and Stubbe has described occult bleeding in the presence of achylia. Elevated blood corticoid levels occur in animals but not in man after ingestion of aspirin, although an enhanced steroid production may be balanced by a more rapid turnover.[27, 28]

The insolubility of salicylates, their caustic action, and the tendency for intact particles to be embedded in the folds of the mucosa suggest the importance of erosive gastroenteritis as a cause. Gastroscopy by Douthwaite and Lintott[29] in 1938, and later by the former author,[30] showed hyperemia and submucous hemorrhages around aspirin particles, but neither Paul[31] nor Caravati and Cosgrove[32] could confirm these findings. Muir and Cossar[22] gave aspirin before gastrectomy was performed, which demonstrated frankly erosive or ulcerative changes in 18 per cent of specimens, and workers in Argentina[33] claimed changes in gastric biopsy specimens after aspirin ingestion.

Although different salicylate preparations have been investigated during gastroscopy,[29, 30] by the presence of occult blood in the stools[6] and gastric aspirate,[34] and by clinical impression[22] no compound can be considered free from the complication of bleeding at present. The method described in this article has been utilized for a comparative study of alimentary bleeding during the administration of several preparations.[18]

It was not the purpose of this communication to assess the clinical significance of low grade bleeding into the gastrointestinal tract, but only to stress the frequency and extent of this blood loss. The entity of salicylate anemia was described by Summerskill and Alvarez[35] in two patients and other patients with a similar disorder were previously described by Kelly[36] and Modell and Paterson.[37] When one considers the enormous quantity of salicylates ingested, the description of a handful of patients seems unimportant, but the very universality of aspirin ingestion may make the consideration of this drug as the etiologic factor in an individual case of unexplained anemia unusual.

Fifty-two per cent of the thirty-five subjects in this study bled 1.8 to 6 ml. per day, averaging 2.8 ml., and 17 per cent bled over 6 ml., averaging 8.8 ml.

per day. The absorption of 1 mg. of iron is required to replace the loss of 2 ml. of blood, so that an average of 1.5 and 4.5 mg. of iron daily were required by these two groups.

The total iron stores of the body when fully saturated have been assessed at 1.5 Gm., so that it would require low grade iron loss of this degree for many months before these stores became exhausted even without considering the reabsorption of iron hemoglobin. However, many individuals who take salicylates chronically have, by reason of their disease, reduced iron stores and, frequently, a reduced dietary intake; the normal individual absorbs only 10 per cent of the 12 to 15 mg. of dietary iron daily. A clinical assessment is made more complex by the work of Roche and Perez-Gimenez[38] who describe reabsorption of 44 per cent of iron from the hemoglobin lost into the gastrointestinal tract in patients with hookworm disease. Although the absorption of inorganic iron in the iron-deficient subject is enhanced, increased absorption from food is denied by Moore and Dubach,[39] except in the presence of excess ascorbic acid. However, disregarding reabsorption of hemoglobin iron, a continuous loss of only 1.5 ml. of blood per day is equivalent to the monthly blood loss in an average menstrual period, and the loss of 6 ml. of blood daily over a nine month period is equivalent to the total iron requirements of a woman throughout pregnancy. This degree of alimentary bleeding may therefore be held responsible for iron deficiency in some patients who take salicylates chronically.

SUMMARY

Quantitative studies of the incidence and magnitude of gastrointestinal bleeding in subjects taking aspirin in which radioactive chromium^{51}-labeled red cells were used revealed that about 70 per cent of the subjects lost blood in amounts which varied up to 13.8 ml. per day, average 4.3 ml. per day. This suggests that alimentary bleeding represents a very frequent side effect of aspirin therapy, and in some patients chronic ingestion of salicylates may be accompanied by sufficient blood loss to induce iron deficiency over a prolonged period.

The factors that may be associated with this type of gastrointestinal bleeding are discussed.

The method described in this communication would appear to be the most accurate available for a comparative study of the extent of alimentary bleeding during the administration of various salicylate compounds.

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ADDENDUM

Since the completion of this study, Matsumoto and Grossman (Proc. Soc. Exper. Biol. & Med. 102: 517, 1959) have reported bleeding into the alimentary tract during aspirin ingestion in amounts comparable to those reported in this communication in an unstated percentage of patients who suffered from gastrointestinal diseases. They found that hourly antacid administration reduced significantly, but did not abolish, the blood loss, and that aspirin in solution gave similar results to the tablet form.

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EFFECT OF MEPERIDINE AND SECOBARBITAL UPON RENAL EXCRETION OF WATER AND SOLUTE IN MAN

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It has been shown that the administration of therapeutic doses of morphine to normal man undergoing water diuresis results in a decreased urine flow that may be attributed to reduction in glomerular filtration rate and solute excretion without any evidence for increased antidiuretic hormone release (ADH). The present study is concerned with the effects of meperidine (parenterally) and secobarbital (orally) upon water diuresis in normal men. In general, drug administration was followed by a decrease in urine flow accompanied by a decrease in endogenous creatinine clearance or total solute excretion, without rise in urinary concentration. The data indicate that the reduction of urine flow consequent to drug administration cannot be attributed to augmentation in the level of ADH activity.

IT has been generally accepted that a decrease in urine flow may follow the administration of sedative and analgesic drugs.[1] This has been commonly attributed to the release of antidiuretic hormone.[2-4] However, Papper and coworkers[5] have shown that the decreased urine flow which follows the administration of therapeutic doses of morphine in normal man undergoing water diuresis may be attributed to reduction in glomerular filtration rate and solute excretion and that there is no evidence for an increased release of antidiuretic hormone.

In the present study the data suggest that meperidine and a short acting barbiturate, secobarbital, like morphine, result in a decreased rate of urine flow not ascribable to an increased release of antidiuretic hormone.

METHODS

The subjects were patients admitted for the treatment of a variety of mild dermatologic disorders without renal, hepatic, cardiac, or endocrine abnormalities. Diet was unrestricted. An hour after a light breakfast an oral water load of 20 ml. per kilogram body weight was established and maintained throughout the experimental period by the ingestion of a sufficient quantity of water after each voiding to maintain body weight at the level obtained after initial water loading. Subjects remained in a recumbent position, except when standing to void.

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TABLE I. EFFECT OF MEPERIDIN

SUBJECT NO.	MAXIMUM FLOW (ML./MIN.)	DOSAGE* (MG.)	ROUTE	ELAPSED TIME† (MIN.)	DECREASE IN URINE FLOW (ML./MIN.)	CONCENTRATION (MOSM./L.)		SOLUTE EXCRETION (μOSM/MIN.	
						CONTROL	DRUG	CONTROL	DRU
1	13.5	100	S.C.	140	3.6	91	68	1,229	67
2	18.4	100	S.C.	63	3.3	66	62	1,220	92
3	18.1	100	S.C.	39	7.0	90	106	1,630	1,15
4	22.5	100	S.C.	68	3.0	66	64	1,490	1,25
5	12.9	100	I.M.	42	2.8	48	50	620	52
6	13.2	100	I.M.	38	2.4	62	60	817	64
7	20.2	100	I.M.	49	2.3	58	63	1,170	1,15
8	19.0	100	I.M.	62	2.1	75	85	1,427	1,43
9	12.0	100	I.M.	43	3.8	72	87	864	71
10	12.0	100	S.C.	35	2.4	64	64	768	67
11	12.1	160	S.C.	37	4.9	71	89	863	64
12	11.9	126	S.C.	58	2.8§	60	60	714	54
13	12.7	120	S.C.	53	2.5	74	74	940	85
14	21.5	165	S.C.	32	2.5	53	59	1,085	1,11
15	15.0	185	S.C.	47	9.4	73	124	1,164	68
16	17.8	122	S.C.	32	2.8	42	43	745	65

S.C. = subcutaneous; I.M. = intramuscular.

*Subjects 11 to 16 received 2.2 mg. meperidine per kilogram body weight.

†Time from the administration of meperidine to the end of the first collection period in which decrease in rate of urine flow of at least 2 ml. per minute occurred.

‡Inulin and PAH clearances decreased from 106 and 483 ml. per minute, respectively, to 96 and 4 ml. per minute.

§Within 180 min. flow rose to control levels with rise in solute excretion to 649 μOsm per minute a C_{cr} to 107 ml. per minute; however, urine osmolality remained unchanged (59 mOsm. per liter).

After the onset of diuresis, urine specimens were collected at intervals of 30 minutes or less. After the establishment of maximal urine flow, meperidine hydrochloride in a single dose of 100 to 185 mg. was administered subcutaneously or intramuscularly to 16 subjects (Nos. 1 to 16) and a single oral dose of 200 mg. of sodium secobarbital was administered orally to 10 subjects (Nos. 17 to 26). Urine collections were continued at intervals for several hours.

Venous blood samples were drawn at the time of maximal urine flow immediately prior to drug administration, and again at the end of the experiment. Blood and urine were analyzed for sodium, potassium, chloride, and creatinine by methods previously described from this laboratory.[6] Total osmolality was determined by freezing point measurement with a Fiske osmometer. Calculation of osmolar and free water clearance were based on equations provided by Smith.[7]

Blood pressure and heart rate were recorded approximately midway between urine collections.

RESULTS

A. *Meperidine (Table I, Fig. 1).*—

1. All subjects experienced drowsiness or dizziness or both within 7 to 25 minutes after drug administration. These symptoms persisted for at least one hour in a majority of subjects. Blood pressure and pulse remained stable in 14 subjects, but two (Nos. 3 and 8) experienced postural hypotension.

2. Within 32 to 68 minutes after the administration of meperidine, urine flow diminished by 2.1 to 9.4 ml. per minute in 15 of the 16 subjects (Nos. 2 to 16) (Table I, Fig. 1). In the remaining subject (No. 1), antidiuresis did not occur until after the second hour.

ON MAINTAINED WATER DIURESIS

SODIUM (μEq/MIN.)		POTASSIUM (μEq/MIN.)		CHLORIDE (μEq/MIN.)		CLEARANCES (ML./MIN.)					
						CREATININE		OSMOLAR		FREE WATER	
CONTROL	DRUG	CONTROL	DRUG	CONTROL	DRUG	CONTROL	DRUG	CONTROL	DRUG	CONTROL	DRUG
355	118	130	70	359	133	148	149	4.5	2.5	9.0	7.4
198	144	173	114	248	182	167	154	4.4	3.4	14.0	11.7
357	241	202	122	398	264	203	179	6.0	4.4	12.1	6.7
254	196	163	135	267	230	184	185	5.3	4.5	17.2	15.0
48	39	74	55	48	34	131	107	2.2	1.8	10.7	8.3
132	100	104	78	159	120	164	150	3.0	2.4	10.2	8.4
210	228	104	74	242	242	173	167	4.2	4.1	16.0	13.8
403	327	183	154	387	357	168	183	5.2	5.3	13.8	11.6
128	90	106	92	164	145	146	131	3.1	2.6	8.9	5.6
156	123	125	94	170	123	135‡	124‡	2.6	2.3	9.4	7.3
107	64	135	96	157	113	124	117	3.1	2.7	9.1	4.8
130	62	63	52	131	77	103	100	2.6	2.0	9.3	7.1
123	78	131	89	118	72	126	116	3.4	2.8	9.3	7.4
151	139	268	226	222	185	170	179	3.9	4.0	17.6	15.0
231	126	98	73	232	167	156	124	4.2	2.5	10.8	3.1
132	122	130	102	164	145	140	123	2.8	2.5	14.9	12.5

3. Endogenous creatinine clearance varied by less than 10 ml. per minute in six subjects, rose by 15 ml. per minute in one subject (No. 8), and decreased by 10 to 32 ml. per minute in the remaining nine subjects.

4. Total solute excretion decreased by 40 to 554 μOsm per minute in 14 subjects and rose slightly in the other two. Urine osmolality increased by 10 to 18 mOsm. per liter in four subjects and by 51 mOsm. per liter in a fifth subject when urine flow declined. In three of the five (Nos. 3, 8, and 11) this increased concentration was transient. In the other two subjects (Nos. 9 and 15) who experienced severe vertigo and vomiting, respectively, this increase in urine osmolality was sustained. In Subject 1, urine osmolality decreased by 23 mOsm. per liter when urine flow declined. In the remaining 10 subjects, decreased urine flow was not associated with significant change in urinary concentration.

5. The rate of sodium and chloride excretion decreased after drug administration in 15 of the 16 subjects. In all subjects there was a decreased rate of excretion of potassium.

B. *Secobarbital (Table II, Fig. 2).*—

1. Nine subjects (Nos. 17 to 25) experienced definite drowsiness within 15 to 50 minutes after the drug was given. This lasted 55 to 85 minutes. One subject (No. 26) failed to experience or demonstrate any sedative action after the ingestion of secobarbital. Blood pressure and pulse rate remained essentially unchanged throughout the experimental period in all subjects.

2. Within 45 to 96 minutes after the administration of secobarbital, urine flow decreased by 2.0 to 5.6 ml. per minute in eight subjects (Nos. 17 to 24). In two subjects (Nos. 25 and 26) one of whom failed to develop drowsiness, urine flow did not decrease.

3. In six (Nos. 17 to 20, 23, and 24) of the eight subjects whose urine flow declined after drug administration, there was an associated reduction in

TABLE II. EFFECT OF SECOBARBITAL

SUBJECT NO.	MAXIMUM FLOW (ML./MIN.)	ELAPSED TIME* (MIN.)	Δ URINE FLOW (ML./MIN.)	CONCENTRATION (MOSM./L.) CONTROL	CONCENTRATION (MOSM./L.) DRUG	SOLUTE EXCRETION (μOSM./MIN.) CONTROL	SOLUTE EXCRETION (μOSM./MIN.) DRUG
Subjects With Decreased Urine Flow After Drug Administration.—							
17	17.7	62	2.0	56	51	998	790
18	19.5	51	3.4	45	48	878	77[illegible]
19	19.1	45	5.6	61	62	1,169	83[illegible]
20	13.7	78	2.2	58	43	792	49[illegible]
21	18.9	69	2.3	72	75	1,360	1,29[illegible]
22	17.3	96	2.0	44	45	757	68[illegible]
23	16.5	56	3.5	60	62	990	80[illegible]
24	19.6	78	3.0	37	41	729	70[illegible]
Subjects With Increased Urine Flow After Drug Administration.—							
25	17.4	50	1.7	51	54	885	1,02[illegible]
26	15.6	41	2.3	43	48	673	84[illegible]

*Time from the administration of secobarital to the end of the first urine collection period in which a decrease in rate of urine flow of at least 2 ml. per minute occurred (Subjects 17 to 24).

endogenous creatinine clearance of 8 to 22 ml. per minute, whereas in the remaining two subjects creatinine clearance remained unchanged.

4. The reduced urine flow observed in eight subjects after drug administration was accompanied by a decrease of 28 to 333 μOsm per minute in the rate of excretion of total solutes. In no instance was there a significant rise in urinary concentration. In the two subjects (Nos. 25 and 26) who failed to have a decreased urine flow after drug administration, there was an increase

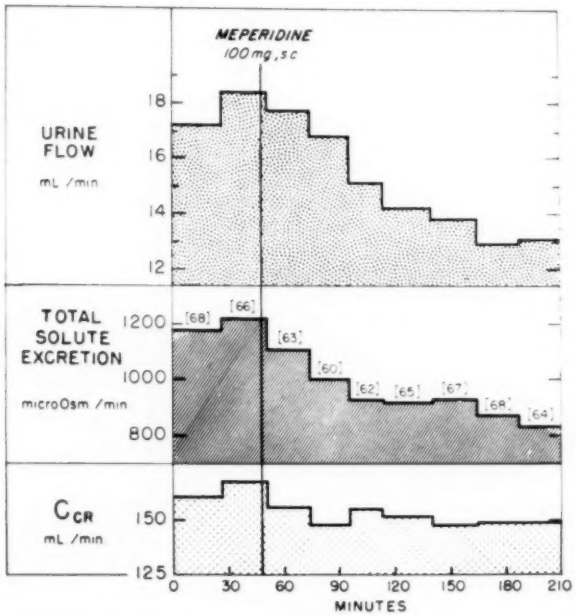

Fig. 1.—The renal response to meperidine in Subject 2. The numbers in brackets indicate urinary concentration in milliosmoles per kilogram of water.

ON MAINTAINED WATER DIURESIS

SODIUM (μEQ/MIN.)		POTASSIUM (μEQ/MIN.)		CHLORIDE (μEQ/MIN.)		CLEARANCES (ML./MIN.)					
						CREATININE		OSMOLAR		FREE WATER	
CONTROL	DRUG	CONTROL	DRUG	CONTROL	DRUG	CONTROL	DRUG	CONTROL	DRUG	CONTROL	DRUG
43	49	161	116	142	115	156	146	3.6	2.9	14.1	12.7
111	77	126	116	160	132	154	142	3.2	2.8	16.3	13.4
330	198	95	87	305	206	132	110	4.2	3.0	14.9	10.5
186	151	63	55	193	153	91	79	2.9	1.8	10.8	9.7
324	289	254	211	402	392	137	138	4.8	4.6	14.1	12.0
119	115	92	83	152	127	162	164	2.8	2.5	14.4	12.8
80	82	120	81	140	141	147	125	3.6	2.9	12.9	10.1
64	61	89	99	120	140	140	124	2.4	2.5	17.0	14.3
133	158	98	142	137	192	131	128	3.1	3.6	14.4	15.5
65	102	68	105	54	90	116	116	2.4	3.0	13.2	14.9

in the rate of total solute excretion without significant alteration in endogenous creatinine clearance.

5. The rate of sodium excretion decreased significantly after drug administration in four of the eight subjects whose rate of urine flow decreased, and changed little in the other four subjects. The two subjects in whom diuresis continued after drug administration had an increase in sodium excretion.

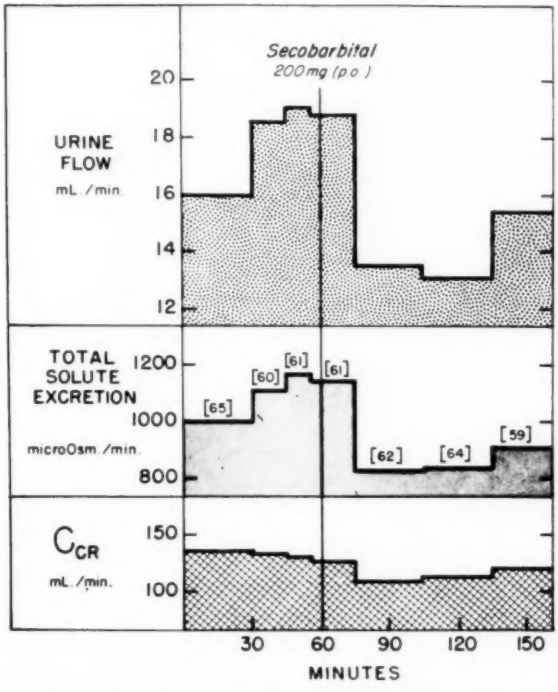

Fig. 2.—The renal response to secobarital in Subject 19. The numbers in brackets indicate urinary concentration in milliosmoles per kilogram of water.

DISCUSSION

The administration of a therapeutic dose of meperidine or secobarbital was in general followed by a reduction in the rate of urine flow in subjects undergoing maximal water diuresis. This antidiuresis was uniformly accompanied by a decreased rate of excretion of total solutes in the secobarbital experiments and in all but two of the studies by a reduction in endogenous creatinine clearance. The decreased rate of urine flow after administration of meperidine was generally associated with a reduction in the rate of total solute excretion and, in nine instances, by a definite fall in endogenous creatinine clearance.

In each experiment in which secobarbital was administered and in most of the studies in which meperidine was employed the observed reduction in urine flow was not associated with increased urinary concentration, an observation which provides strong evidence against augmentation of antidiuretic hormone activity. Although increased urinary concentration does not in and of itself necessarily indicate enhanced antidiuretic hormone activity, in our present state of knowledge one cannot implicate antidiuretic hormone as a cause of decreased urine volume except when there is an increase in the concentration of the urine.[8]

The lack of evidence implicating increased antidiuretic hormone activity as a cause of the reduction in urine flow under these conditions necessitates consideration of the role of alterations in glomerular filtration rate (GFR) and in solute excretion. Indeed, there are observations in animals and man which suggest that barbiturate drugs and meperidine under certain circumstances may result in diminished glomerular filtration rate.[3, 9, 10, 11] Whereas an obvious decline in endogenous creatinine clearance was not invariably observed in association with the reduction in urine flow in the present studies, a small but significant decrease occurred in certain individuals. It is conceivable that the drugs produced changes in tubular handling of solutes independent of any action on glomerular filtration rate. This seems less likely, at least in the case of secobarbital, since decreased sodium excretion was a less consistent finding than diminution in total solute excretion.

If secobarbital and meperidine result in antidiuresis via their action on renal circulation, this effect could be primarily in the kidney or secondary to a change in cardiac output. Most of the available information suggest that sedative doses of these drugs may not alter cardiac output,[12, 13] although the possibility of such effect cannot be entirely dismissed.[14, 15] The present studies offer no evidence on this point.

CONCLUSIONS

1. In the course of sustained water diuresis, 16 subjects were given meperidine parenterally and 10 subjects were given secobarbital orally.

2. In general, the administration of these drugs was followed by a decrease in the rate of urine flow.

3. With few exceptions the reduction in the rate of urine flow was not accompanied by a rise in urinary concentration.

4. In general, the decreased water diuresis was accompanied by an observed decrease in endogenous creatinine clearance or total solute excretion or both.

5. The reduction of urine flow observed after drug administration under these conditions cannot be attributed to augmentation in the level of antidiuretic hormone activity, but is consistent with a hemodynamic action of the drugs.

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PLASMA VOLUME EXPANSION AND REACTIONS AFTER INFUSION OF AUTOLOGOUS AND NONAUTOLOGOUS PLASMA IN MAN

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Blood plasma is usually considered to be the ideal plasma volume expander and its use has generally been considered to be without adverse effect. Recent studies in dogs have indicated that such is not always the case. Studies were therefore undertaken in man to determine the incidence of reactions and the effect upon plasma volume expansion of infusions of autologous plasma, nonautologous plasma, and dextran. Autologous plasma was found to produce the anticipated increase in circulating plasma volume and did not give rise to reactions. Similar results were obtained with the infusion of dextran. However, nonautologous plasma resulted in marked cutaneous and systemic reactions in a significant number of cases. The plasma volume expansion was often less than anticipated, and among those who developed severe reactions an actual contraction of plasma volume was found.

ERYTHROCYTE incompatibility reactions have been widely studied for several decades. Typing and cross match techniques have been developed to decrease or prevent such reactions. More recently reactions to the leukocytes of the buffy coat of whole blood have been demonstrated.[1] However, to date no well-defined system of leukocyte antigens has been uncovered. For many years sporadic reports have suggested that there is an immunologic specificity of plasma proteins and that one individual may be hypersensitive to the plasma proteins of another individual of the same species.[2-5] Allergic reactions to plasma infusions have been reported in the literature.[3, 7-9] However, it is widely held that plasma is an ideal volume expander and that its use is free of significant allergic reactions. Recent animal studies by Bliss and associates[10-13] lead us to reconsider this opinion. They have shown that dogs react more strongly to plasma from other dogs (nonautologous plasma) than to their own (autologous plasma). Intravenous infusions of nonautologous plasma often resulted in severe urticarial reactions and the plasma volume expansion in such dogs was much less than anticipated. Autologous plasma given in a similar manner did not give rise to such reactions and consistently gave good plasma volume expansion.

If similar incompatibility reactions should occur frequently in man, the clinical significance is apparent. Studies were therefore undertaken to determine whether significant reactions to nonautologous plasma transfusions occur in man.

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MATERIALS AND METHODS

The subjects used in this study were healthy young volunteers or young hospitalized patients who were free of cardiovascular, dermatologic, or other disease likely to influence blood volume dynamics and capillary permeability. Plasma for the study was collected in silicone coated bottles which contained disodium citrate, 2 Gm., in 30 ml. of water. About 550 ml. of blood was drawn and immediately centrifuged at 1,700 r.p.m. at a temperature of 6° C. for 30 minutes. The plasma was then removed using a closed system and stored at −23° C. until used a few days later. Blood for skin testing was drawn immediately prior to use into sterile vacuum tubes that contained 0.75 mg. of heparin for each 10 ml. of blood. These were centrifuged and the plasma was removed under sterile conditions.

Ten individuals received about 500 ml. of autologous plasma. Fifteen persons received nonautologous plasma from a donor of the same erythrocyte blood group. Nine of these had previously acted as recipients of autologous plasma. These nine each received blood from a single donor whereas the blood used in the other six nonautologous cases was obtained from two or three donors.

One pair of identical twins (confirmed by erythrocyte genotyping) was included among the recipients of autologous and nonautologous plasma, the plasma of each one being given to the other twin in the nonautologous phase of the experiment.

An additional nine individuals received 500 ml. of a 6 per cent dextran in saline solution. The mean molecular weight of the dextran was 75,000 and 90 per cent of the particles ranged in molecular weight from 25,000 to 200,000.

Five patients were used as control subjects. The first was given a transfusion of 600 ml. of normal saline with 2 Gm. of disodium citrate whereas the other four were given only sufficient saline to maintain patency of the catheter used for infusion.

All sampling and infusions were carried out through a small caliber polyethylene catheter inserted into the antecubital vein using a three-way stopcock system and a continuous flow of saline to keep the catheter patent.

Plasma volume determinations were performed using radioactive iodinated serum albumin.* Five or 10 μc was used for each determination. This was contained in 10 ml. of saline and delivered through a calibrated syringe connected to the infusion catheter. The syringe was rinsed with saline after each injection. The counting standard was prepared using the same syringe. Blood samples were withdrawn through the catheter 5, 10, 15, and 20 minutes after injection of the radioactive iodinated serum albumin. After insertion of the polyethylene catheter the first plasma volume determination was carried out. Then the plasma or dextran was infused over a 30 minute period by means of a constant flow infusion pump. One hour after completion of the infusion, the plasma volume was redetermined.

Triplicate hematocrit determinations using Wintrobe tubes were made at the time of the blood volume measurements and in some individuals serial hematocrit estimations were carried out throughout the experiment.

All recipients were observed for a period of 2 hours for the presence of allergic type reactions to the infused plasma. An allergic type reaction was considered to be one which was characterized by the presence of urticaria, fever, chills, nasal stuffiness, or dyspnea either singly or in combination.

Skin testing was performed by intradermal injection, at random sites in the back, of 0.1 ml. of plasma. In the earlier studies this was done during the plasma transfusion but in later cases skin tests were carried out before the infusion. To make the reading of the skin tests easier, the subjects were given intravenously either 50 mg. Evans blue dye (T 1824) or 200 mg. Coomassie blue dye. When administered during the infusion of plasma the dye also delineated any urticaria that appeared. The size of the wheal that resulted from the skin test was measured by two observers.

The plasma protein was determined at the same time as the plasma volume. In the earlier experiments the biuret method was used but in the later cases and in those where

*Supplied by Charles E. Frosst and Company.

TABLE I

EXPERIMENT NO.	NO. OF DONORS	EXPERIMENT NO. OF DONOR	BLOOD GROUP	PLASMA VOLUME INDEX*	HEMATOCRIT BEFORE	HEMATOCRIT AFTER	HEMATOCRIT INDEX†	PROTEIN CONCENTRATION BEFORE	PROTEIN CONCENTRATION AFTER	REACTIONS SKIN	REACTIONS SYSTEMIC	DIFFERENCE IN SIZE BLUE WHEAL (MM.)	RECIPIENT ALLERGY HISTORY
Autologous Plasma.—													
14				+123	45.75	44.00	+ 88			—	—		
15				+ 67	40.75	38.75	+ 69	7.05	6.93	—	—		
18				+ 63	43.80	40.20	+134	6.66	6.50	—	—		
20				+ 96	41.57	37.62	+119	5.92	5.95	—	—		
25				+121	39.41	35.46	+123	5.97	5.91	—	—		
26				+ 61	41.15	37.57	+ 94	5.96	5.96	—	—		
38				+ 74	49.57	45.08	+104	7.31	7.19	—	—		
41				+ 94	46.03	42.47	+106	6.30	6.70	—	—		
44				+ 82	43.05	40.43	+ 81	6.55	6.43	—	—		
45				+ 50				6.59	6.59	—	—		
Mean				+ 83			+102						
Nonautologous Plasma.—													
1	2		B+	*−260*						local	—	7.5	neg.
2	3		B+	+ 17						—	—		neg.
8	3		O+	+ 60	41.00	39.00	+ 70	6.56	6.85	local	—	8.0	neg.
9	3		A−	+ 86	47.00	44.25	+ 73	6.98	7.38	—	—	0.0	neg.
11	2		O+	+101	41.00	38.00	+ 89	7.02	7.02	—	—	5.5	pos.
13	3		A+	+ 39	45.25	41.75	+ 97	6.83	7.20	generalized	chills, headache	3.0	neg.
17	1	15	A−		41.50	42.00	*− 17*	7.31	7.88	generalized	chills	7.0	pos.
22	1	18	O+	+ 99	44.33	41.54	+ 83	6.41	6.35	—	—	5.0	pos.
23	1	20	O+	*− 20*	44.07	46.34	*− 63*	6.90	6.90	generalized	nasal, joint pains	5.0	pos.
28	1	26	O+	+ 70	38.85	36.60	+ 65	6.22	7.07	—	—		neg.
29	1	25	O+	*−130*	40.93	45.00	*−150*	7.19	6.95	generalized	chills, headache, faintness		neg.
42	1	38	A+	+ 62	44.22	42.71	+ 45	6.77	7.21	—	—		neg.
43	1	41	A+	+ 43	44.13	42.50	+ 49	6.50	6.77	—	—		neg.
Mean				+ 14			+ 31						
46‡	1	45	A−	+ 35	39.80	38.36	+ 44	6.30	6.55	—	—	5.0	neg.
47‡	1	44	A−	+ 52	40.96	38.67	+ 68	6.30	6.54	—	—	0.0	neg.

Dextran.—								
7	+118	44.00	40.50	+ 96	7.44	6.73	—	—
10	+ 94	48.00	43.80	+108	7.63	6.54	—	—
21	+108	44.77	40.27	+134	6.98	5.86	—	—
30	+118	38.81	35.35	+105	7.01	5.92	—	—
31		43.82	39.46	+159	6.81	6.10	—	—
33	+ 63	43.50	41.15	+ 68	6.95	6.11	—	—
34	+ 77	44.91	41.51	+ 96	7.02	6.38	—	—
35	+ 49	51.22	47.89	+ 83	7.33	6.30	—	—
40	+ 32	37.65	35.27	+ 83	7.39	6.72	—	—
Mean	+ 82			+104				

*Plasma volume index $= \dfrac{\text{final volume} - \text{initial volume}}{\text{infusate} - \text{plasma withdrawn in sampling}} \times 100.$

†Hematocrit index $= \dfrac{\text{actual hematocrit change}}{\text{predicted hematocrit change}} \times 100.$

‡Identical twins.

dextran was given, the micro Kjeldahl method was employed. Samples of the donor plasma were measured for protein content.

The blood pressure and leukocyte count were determined before and after infusion. No significant change was observed.

RESULTS

In the five individuals who served as control subjects there was a moderate increase in plasma volume of 70 to 171 ml. (mean 116 ml.). The changes in those receiving plasma or dextran are shown in Table I. In order to allow for the different volumes of plasma transfused, changes in plasma volume were also evaluated as a plasma volume index. This value represents percentage change in plasma volume related to the volume of the plasma or dextran infused, i.e.,

$$\text{Plasma volume index} = \frac{\text{final volume} - \text{initial volume}}{\text{infusion volume} - \text{plasma withdrawn in sampling}} \times 100$$

We have classified the results as good expansion (index greater than plus 50 per cent), poor expansion (index positive but less than plus 50 per cent), and contraction (index negative).

All the subjects who received a reinfusion of their own plasma had a good expansion, but when they received plasma from another donor only half showed good expansion and three showed contractions of 20 to 260 per cent. Dextran gave a good expansion in seven individuals of the nine studied. The results are summarized in Table II. It can be seen that autologous plasma gave a mean plasma volume index of 83 per cent. Dextran gave a mean plasma volume index of 82 per cent, but nonautologous plasma a plasma volume index of only 18 per cent.

TABLE II. PLASMA VOLUME CHANGES AFTER INFUSION OF AUTOLOGOUS PLASMA, NONAUTOLOGOUS PLASMA, AND DEXTRAN

	AUTOLOGOUS PLASMA (9 EXPERIMENTS)	NONAUTOLOGOUS PLASMA (15 EXPERIMENTS)	DEXTRAN (9 EXPERIMENTS)
1. *As Determined by the Plasma Volume Index.*—			
Good expansion	9	7	6
Poor expansion	0	4	2
Contraction	0	3	0
Results not available	0	1	1
Mean plasma volume index	83%	18%	82%
2. *As Determined by the Hematocrit Index.*—	(10 EXPERIMENTS)		
Good expansion	9	7	9
Poor expansion	0	3	0
Contraction	0	3	0
Results not available	1	2	0
Mean hematocrit index	83%	35%	104%

The identical twins had plasma volume indices of 82 and 50 per cent, respectively, when they received a reinfusion of their own plasma, but the indices were 52 and 35 per cent when they received plasma from the other twin.

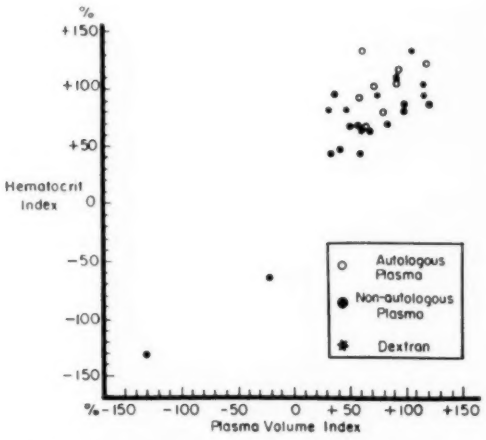

Fig. 1.—Correlation of plasma volume index with hematocrit index. Includes only those experiments where both indices are available.

Hematocrit Changes.—In the four control subjects a mean increase of 0.33 per cent in hematocrit occurred. In the subjects transfused with plasma or dextran a good correlation was found with the plasma volume changes. In order to make quantitative comparisons between hematocrit changes and plasma volume, a hematocrit index was calculated based on the predicted hematocrit change. It was assumed that there was complete retention of the infusate within the circulation and that the venous hematocrit reflected the whole body hematocrit. Previous animal experiments showed that this latter assumption did not introduce appreciable error in calculating differential changes in red cell mass.[10] The hematocrit index then is the actual hematocrit change expressed as a percentage of the predicted hematocrit change. As with the plasma volume changes, the hematocrit indices were classified as good expansion (index greater than plus 50 per cent, poor expansion (index positive but less than plus 50 per cent), and contraction (index negative).

The results from calculations of the plasma expansion from the change in hematocrit were in satisfactory agreement with those determined from the plasma volume, particularly in view of the relatively small changes produced by infusion of 400 to 500 ml. of plasma or dextran (Fig. 1). Once again the effects of autologous plasma (hematocrit index 83 per cent) and dextran (hematocrit index 104 per cent) showed good expansions whereas nonautologous plasma was poor (hematocrit index 35 per cent) (Table II). It should be noted that the poorer expansion of the identical twins when receiving plasma from the other twin as compared with their own plasma was confirmed by this method.

Serial hematocrit determinations form a simple way of following the course of plasma expansion during and after an infusion. In Fig. 2, the results of three such experiments are shown. In Experiment 41 when 449 ml. of autologous plasma was infused there was a steady fall of the hematocrit during

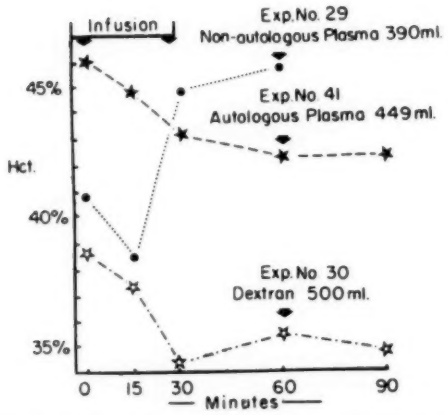

Fig. 2.—Serial hematocrit changes during infusion of autologous plasma, nonautologous plasma, and dextran.

the infusion which remained constant at the reduced level after the infusion was over. A similar result was obtained in Experiment 30 when 500 ml. of dextran was infused. On the other hand, Experiment 29 in which 390 ml. of nonautologous plasma was infused gave different results. After 15 minutes, the hematocrit had fallen to the expected degree, but during the second 15 minutes of the infusion the hematocrit change reversed, and at the end of infusion the hematocrit was 4 per cent higher than initially. Thirty minutes later a small further increase in hematocrit had occurred. This experiment was one in which contraction of plasma volume occurred (−500 ml.) and the increase in the hematocrit in the second 15 minutes coincided with the development of reactions (generalized urticaria, headache, faintness, chills).

Plasma Protein Changes.—Calculation of total circulating plasma protein was relatively inaccurate as the inaccuracies of plasma protein determination (± 2 per cent) and plasma volume determination (± 2 to 3 per cent) were compounded. But the changes were sufficient to give clear indications of the nature of the processes that were occurring as a result of transfusion. In the autologous group, no significant change in plasma protein concentration occurred as a result of transfusion (Table III). In the nonautologous group, however, a small but significant increase in protein concentration occurred after transfusion which demonstrated a greater loss of fluid than of protein from the circulation. Nevertheless, in those cases in which a reduction in plasma volume was seen after transfusion, a considerable loss of protein from the circulation had presumably occurred. With dextran a highly significant reduction in plasma protein concentration occurred as anticipated.

Reactions.—Reactions were divided into two groups. The first, designated as skin reactions, consisted of urticaria or angioedema. The second group consisted of individuals who, in addition to skin reactions similar to group one, complained of chills, headache, nasal stuffiness, faintness, or joint pains. These systemic symptoms occurred in various combinations.

Urticaria occurred in six individuals who received nonautologous plasma. In two it consisted of a few scattered wheals whereas, in the other four, giant

urticaria was noted. In three cases, the giant urticaria was confluent and covered a large portion of the body area. All four individuals with generalized urticaria had systemic symptoms. In one, joint pains occurred several hours after the infusion. A definite correlation existed between the occurrence of cutaneous and systemic reactions and the plasma volume expansion which is shown in Table IV.

A personal history of allergy was obtained in four of the 15 individuals who received nonautologous plasma. Although only four patients had generalized urticaria and sytemic reactions, these included two of the four individuals with an allergic history. Although a relationship may exist the results are not statistically significant.

There were no reactions to autologous plasma or dextran.

Skin Tests.—The results of the skin tests are reported in Table I. They are expressed as the difference in size (in millimeters) of the blue whealed areas that resulted from the autologous and nonautologous plasma. Results are available in ten of the fifteen individuals who received infusions of nonautologous plasma. All but two obtained a larger wheal from nonautologous than autologous plasma. The remaining two had reactions of similar degree. No patient reacted more strongly to autologous than nonautologous plasma. The average diameter of the nonautologous plasma skin reactions exceeded the average diameter of the autologous plasma skin reactions by 4.5 mm. A significant correlation did not appear to exist between the degree of the skin test reaction to nonautologous plasma and the plasma volume index after infusion of the same nonautologous plasma intravenously. However, the number of tests performed was small. A larger series of skin tests in which autologous and nonautologous plasma were used is reported.[14]

TABLE III. PLASMA PROTEIN CONCENTRATIONS

	BEFORE INFUSION	AFTER INFUSION	P ("T" TEST)
Autologous plasma	6.48 ± 0.47* (9)†	6.46 ± 0.49	0.8
Nonautologous plasma	6.79 ± 0.34 (11)	7.05 ± 0.44	0.03
Dextran	7.17 ± 0.79 (9)	6.30 ± 0.29	< 0.01

*Standard deviation.
†Number of subjects.

TABLE IV. CORRELATION OF PLASMA VOLUME EXPANSION BY NONAUTOLOGOUS PLASMA TO INCIDENCE OF REACTIONS

	GOOD EXPANSION	POOR EXPANSION	CONTRACTION
Presence of skin reactions			
6 experiments	1	1*	4
Presence of systemic reactions			
4 experiments	0	0	4

*As determined by plasma volume index. The hematocrit index suggested a good expansion.

Report of a Typical Reaction to Nonautologous Plasma.—The subject was a healthy young doctor of the blood group O Rh+ with a history of seasonal allergic rhinitis. He first received a transfusion of 542 ml. of his own

plasma. He showed a full plasma volume expansion; the protein was retained within the circulation; the hematocrit fell to the extent expected, and there were no cutaneous or systemic reactions. Next he received 539 ml. of nonautologous plasma obtained from a single donor of group O Rh^+. The plasma volume in this experiment fell by 100 ml.; the hematocrit rose, and the circulating plasma protein decreased. Severe urticaria developed toward the end of the infusion and became maximal within 30 minutes after its completion. Fig. 3 shows the degree of the skin reaction, which is well defined by the Evans blue dye (T 1824). Chills were noted during the urticarial reaction and about 12 hours later he complained of joint pain. His symptoms disappeared after the administration of epinephrine and diphenhydramine. Details of the volume changes are summarized in Table V.

TABLE V. DATA ON EXPERIMENT 23, DR. L. R.

DETERMINATION	AUTOLOGOUS PLASMA	NONAUTOLOGOUS PLASMA
Plasma volume before infusion	3,431 ml.	3,191 ml.
Volume plasma infused	542	539
Plasma volume after infusion	3,920	3,089
Change in volume	+489	−102
Plasma volume index	+ 96%	− 20%
Total plasma protein before infusion	203 Gm.	220 Gm.
Protein infused	35	41
Protein after infusion	233	213
Change in circulating plasma protein	+ 30	− 7
Hematocrit before infusion	41.6%	44.1%
Hematocrit after infusion	37.6	46.3
Hematocrit index	+119%	− 63%
Skin tests	negative	positive
Skin reaction	no	yes
Systemic reaction	no	yes

DISCUSSION

The results obtained in the present investigation indicate that cutaneous and systemic reactions to nonautologous plasma are common under the conditions of the experiment, and that plasma volume expansion is usually poor when such reactions occur. Even in the absence of obvious skin and systemic reactions, nonautologous plasma often produces a less satisfactory plasma volume expansion than occurs with autologous plasma or dextran.

Allergic type reactions to plasma infusions have been reported previously. Levine and State[3] reported an 80 per cent incidence of reactions to nonautologous plasma infusions among a group of individuals who showed positive skin tests to the same plasma. Urticaria was a feature of all the reactions, whereas headache, dyspnea, epigastric distress, chills, and fever occurred in some. They did not note reactions among those with negative skin tests. Weinstein[7] reviewed 1,500 plasma infusions and reported a total of 15 reactions. Of these, 11 were febrile in type and one individual was reported to have had urticaria and slight dyspnea. The results of 10,000 pooled liquid plasma infusions were studied by Miller and Tisdall in 1945.[9] Two hundred and ninety-six reactions occurred,

of which 105 were of the allergic type. The allergic type reactions were urticarial except for three who had asthmatic symptoms. These reactions, like those reported in the present article, occurred during or immediately after the infusion.

Many explanations have been advanced to explain these reactions. Earlier writers speculated that isoagglutinins in the infused plasma might be responsible.[3] However, there has been no satisfactory evidence to substantiate this thesis. Isoantibody reactions should not occur if the donor's blood is of the same ABO erythrocyte group as the recipient's. Immune erythrocyte antibody reactions are unlikely when young male donors without a history of having received blood transfusions are used. Thus this argument does not seem a valid explanation of the reactions reported in this article.

In the past, attempts have been made to explain these reactions by the passive transfer of skin-sensitizing antibodies of the atopic type, or the transfer of atopic allergens to which the recipient was already sensitive.[9] Although this hypothesis has been substantiated in a small number of cases, it is unlikely that it would account for the large number of allergic type reactions observed in this study.

Others have attempted to prove the immunologic specificity of plasma proteins or the presence of plasma protein groups.[4] However, the work of Cumley and Irwin[4] suggesting the presence of actual plasma protein groups was not confirmed in a limited study by Goldberg and de Gara.[5] Bernfeld and co-workers[6] studied the individual electrophoretic patterns of human plasma proteins and feel that the pattern for a given individual is specific. Bliss and Stewart[13] studied the cutaneous response of autologous and nonautologous plasma in dogs. Closely inbred dogs were tested with autologous and nonautologous plasma and it was found that such animals reacted to each other's plasma in spite of their close genetic similarity. Thus the plasma from each dog was immunologically specific. Puppies in the neonatal period did not react to nonautologous plasma. The authors suggested that the genetic factors responsible for plasma specificity were located on a large number of chromosomal loci and that, in dogs at least, there was no evidence of plasma protein groups.

A pair of identical twins were included in the present study to see if the plasma of the other twin behaved like nonautologous plasma. Although the skin tests showed no reaction, retention of plasma within the circulation was less in both the twins than after autologous transfusion.

If it is assumed that the nonautologous plasma reactions are due to plasma protein specificity, some consideration of the mechanism of the reaction is required. The reactions occur without prior sensitization to the plasma proteins concerned, and occur within minutes after skin testing or infusion. This reaction differs from the similarly specific mechanism leading to rejection of skin homografts. Here in an individual without previous exposure, a time interval of several days must occur before rejection takes place. Medawar[15] has suggested that cellular antibodies formed against the homograft lead to its eventual rejection.

Fig. 3.—Cutaneous reaction to nonautologous plasma transfusions. The plasma volume changes in this subject are listed in Table V. The dark areas on the chin, lips, and forehead are due to extravasation of T 1824 into the wheals.

There is considerable evidence that the interaction of a substance in nonautologous plasma with some tissue component, perhaps in the mast cells, leads to the release of histamine which is responsible, in part at least, for the skin and systemic manifestations of the reaction. Bliss and Walker[12] have shown, in dogs, a marked rise in free histamine at the sites of intradermal nonautologous plasma injections as compared to autologous plasma. Bliss and Stewart[11] noted as well that the use of tissue histamine depletors prevented skin reactions when nonautologous plasma was subsequently injected. The use of antihistamines also resulted in a marked decrease in the degree of whealing that resulted from nonautologous skin tests. The intravenous infusion of nonautologous plasma[10] produced a marked increase in gastric acidity. This did not occur with autologous plasma and is taken as evidence of histamine release from the tissues.

Although the presence of urticaria is evidence of loss of circulating plasma into the tissue, and the staining of such urticaria with Evans blue dye is evidence of loss of plasma proteins into the wheals, little attention has been paid to the quantitative loss of plasma and plasma proteins that results from such reactions. Wollheim and Schneider[16] carried out plasma volume determinations using Evans blue dye in human beings before and after infusions of nonautologous plasma and whole blood. Of those who received dried or unmodified plasma, less than two thirds had a significant rise in the plasma volume after infusion. They correlated their results with the initial hematocrit and concluded that those patients with low initial hematocrit had a satisfactory increase in plasma volume, whereas those with normal or high hematocrits had a compensatory loss of plasma. It seems more probable that, although their results are

correct, the interpretation was not, and that they in fact were observing a phenomenon similar to that reported here. The concept of overloading of the vascular system leading to an immediate compensatory plasma loss has been popular. In 1958, Huggins and associates[17] gave increments of nonautologous blood to dogs and determined the plasma volume between each. They concluded that after a small infusion of 10 ml. of blood per kilogram or less this mechanism did not operate, but that plasma loss occurred with larger infusions. It seems probable that adequate time was not allowed for plasma loss in the smaller infusions before the plasma volume was redetermined. Thus the significant factor may have been time for the reaction to occur rather than the amount of the infusion.

Guyton and co-workers[18] found a marked loss of plasma after very large infusions of nonautologous plasma which they attributed to increased venous and capillary pressure due to vascular overloading. More recent studies by Remington and Baker,[19] using both autologous and nonautologous plasma, showed that, although an ultimate compensatory loss of plasma may occur after infusion of blood or plasma in the normovolemic animal, this correction does not occur rapidly and a full expansion may be anticipated when autologous blood is used regardless of the size of the infusion. Bliss and collaborators[10] and Remington and Baker[19] show conclusively that full expansion of the plasma volume may be anticipated with sizable autologous plasma or whole blood infusions irrespective of the initial hematocrit. When nonautologous plasma or whole blood is infused the expansion may be less than anticipated in a considerable number of cases and occasionally an actual contraction of plasma volume may occur. Remington and Baker also found that some of their dogs given large nonautologous transfusions died. No clear cause for death was found at autopsy. The results of Deavers and associates[20] suggest that the excess plasma is fairly evenly distributed in the tissues.

Levine and State attempted to predict which individuals would react to infusions of nonautologous plasma by the use of skin testing. In contrast to their results, the results of the present study and those of Bliss and co-workers indicate that this is not a reliable method of prediction.

Although occasional reports of allergic reactions to dextran have been reported,[21] the present study suggests that these are probably not common and that even with dextran of smaller size than that frequently used today, a satisfactory expansion of plasma volume may be anticipated.

The results presented in this paper make it clear that nonautologous plasma transfusions in man, as in the dog, produce frequent reactions that may occasionally be harmful to the patient.

SUMMARY

Human subjects have been infused with autologous plasma, nonautologous plasma, and dextran. Changes in plasma volume were measured directly with iodinated serum albumin and indirectly from the change in hematocrit. Good expansion of plasma volume was found with both autologous plasma and dextran,

and no reactions were seen with either of these. On the other hand, only one half the subjects who were receiving nonautologous plasma showed good expansion of plasma volume and several had a lower plasma volume after transfusion than before. Some of these subjects developed urticaria and systemic reactions. The severity of the reactions was correlated with the poor expansion of plasma volume. On intradermal injection of nonautologous plasma the majority of our subjects showed triple responses but the intensity of these responses was not correlated with the poor expansion of plasma volume or the incidence of transfusion reactions.

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INCREASED BLOOD PRESSURE RESPONSES TO DOPAMINE AND NOREPINEPHRINE PRODUCED BY MONOAMINE OXIDASE INHIBITORS IN MAN

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WITH THE TECHNICAL ASSISTANCE OF ISABELLE AMBROSE

The mechanism by which blood pressure is lowered in patients during treatment with monoamine oxidase (MAO) inhibitors has not been determined. It was of interest to study the cardiovascular effects of various amines before and during treatment with such drugs. Two endogenous amines (norepinephrine and dopamine) which are metabolized by MAO and a synthetic amine (methoxamine) which is not were administered intravenously to hypertensive patients.

The pressor effect of dopamine was markedly augmented and prolonged during MAO inhibition. The pressor effects of norepinephrine and methoxamine were augmented to a lesser degree but were not prolonged, and augmentation occurred only after the development of postural hypotension.

It is concluded that the potentiation of dopamine is directly related to enzyme inhibition, whereas that of norepinephrine and methoxamine is not.

SEVERAL drugs which inhibit monoamine oxidase (MAO) are currently being evaluated clinically, chiefly in the treatment of psychic depression. A frequent side effect of these agents has been the production of postural hypotension. Some have actually been used in the treatment of hypertension.[1, 2] Although there has been much useful speculation relating the central effects of MAO inhibitors to increased tissue levels of naturally occurring amines,[3] such an explanation for the actions on blood pressure presents obvious difficulties.

As an approach to this problem, we have been studying the influence of MAO inhibition on the cardiovascular effects of various amines. The present work in patients with hypertension is concerned with alterations in the blood pressure responses to infusions of norepinephrine, its biochemical precursor, dopamine (3,4-dihydroxyphenylethylamine), and methoxamine. The effects of dopamine were of particular interest since it has been found in sympathetic nerve,[4] is a depressor agent in some species, and apparently has not been administered to humans. Methoxamine was included in these studies because it is a sympathomimetic drug whose structure precludes degradation by MAO.[5]

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TABLE I. DOSES OF AMINES PRODUCING A 25 MM. HG RISE IN SYSTOLIC BLOOD PRESSURE

	BEFORE INHIBITOR THERAPY			DURING INHIBITOR THERAPY				
PATIENT	DOPAMINE MCG./MIN.	NOR-EPINEPHRINE MCG./MIN.	METHOXAMINE MCG./MIN.	DOPAMINE MCG./MIN.	NOR-EPINEPHRINE MCG./MIN.	METHOXAMINE MCG./MIN.	INHIBITOR DOSE MG./DAY	SUPINE MINUS STANDING BLOOD PRESSURE† MM. Hg
G. W.	230	2.5		25	0.7		JB516; 25* 12.5‡	59/20
L. G.	300	0.9		19	0.13		JB516; 25* 6.5‡	77/43
G. W. S.	270	13.0	870	22	2.9	225	JB516; 25	31/16
				100	12.0	960	Nialimide; 200	−5/−17
A. F.	285	5.0	350	27	1.2	120	JB516; 25* 6.5‡	58/29
				85	2.5	180	Nialamide; 200	26/6
				30	1.3	90	Nialamide; 400	48/23
G. S.	320	6.0	330	15	1.3	100	JB516; 25* 12.5‡	47/18
W. C.	315	2.5		17	0.7		SKF-385; 80	45/29
				105	2.4		Nialamide; 200	13/−8
J. O. N.	290	2.4		26	0.9		JB516; 25* 37.5‡	65/48

*Initial dose.
†Obtained from average of four determinations per day on days of amine infusions.
‡Maintenance dose.

METHODS

A comparison was made of the intravenous infusion rates of dopamine* and norepinephrine† which would produce a 25 mm. systolic blood pressure elevation, before and during administration of MAO inhibitors in seven hypertensive patients. Methoxamine hydrochloride‡ was administered similarly to three of the patients. Six of the patients had uncomplicated essential hypertension and the seventh (G. W.) had residual hypertension after surgical removal of a coaretation of the aorta.

Six of the patients were treated with JB-516§ in a dosage of 25 mg. daily and one patient received SKF-385,‖ 80 mg. per day. Each received daily oral dosages of the inhibitor for at least one week and had developed postural hypotension before the amine infusions were repeated. In four of the patients it was necessary to reduce the dosage of the inhibitor because of the marked orthostatic effect; in one patient the dosage of JB-516 had to be increased to 37.5 mg. per day in order to produce pronounced postural hypotension. Infusion studies were repeated in three patients during treatment with another MAO inhibitor, nialamide,¶ in a dosage of 200 mg. per day and additional observations were made at a dosage level of 400 mg. per day in one of these patients. A period of 6 weeks or more intervened between cessation of previous MAO therapy and institution of nialamide dosage.

The 24 hour urinary excretion of tryptamine was determined during MAO inhibitor therapy by the method of Sjoerdsma and co-workers[6] in all patients except G. W. and L. G. With each inhibitor tryptamine excretion exceeded 500 μg per 24 hours, verifying the presence of pronounced MAO inhibition.

All amines were administered in 0.9 per cent sodium chloride solutions. The dose of each amine was calculated as the base. The subjects were kept in bed for at least 30 minutes until stable blood pressure readings were obtained. Then the amine solutions were delivered at predetermined rates for 15 minute periods by means of a constant infusion pump. Blood pressure was determined at 2 minute intervals by the same observer by means of a standard arm-cuff sphygmomanometer. Several infusions of each amine were given to the patients on at least 2 different days before and during therapy with the inhibitors. A dose-response curve was constructed and the dose that produced a 25 mm. Hg increment in systolic pressure was approximated from this curve.

In one of the patients, repeated daily observations of the pressor responses to intravenous infusions of dopamine, 300 mcg. per minute, and norepinephrine, 2.5 mcg. per minute, were also made. The amine infusions were started one week before institution of therapy and were continued for 4 weeks during the course of therapy.

RESULTS

The rates of amine infusions that produced a 25 mm. systolic blood pressure rise before and during treatment with MAO inhibitors as well as the magnitude of the postural hypotension produced by the various inhibitors are shown in Table I.

Intravenous infusions of dopamine at rates of 230 to 325 mcg. per minute for 15 minutes were required to produce increments in systolic pressure of about 25 mm. Hg in the different hypertensive patients. Diastolic pressure and heart rate were usually not altered significantly. The patients frequently described a more forceful heart beat during these infusions, but had no other

*Dopamine-HCL, California Research Institute.
†l-Norepinephrine bitartrate, Winthrop Laboratories.
‡Vasoxyl hydrochloride, Burroughs Wellcome & Co., Inc.
§Catron, 1-phenyl-2-hydrazinopropane hydrochloride, Lakeside Laboratories, Inc.
‖Trans dl-2-phenylcyclopropylamine hydrochloride, Smith Kline & French Laboratories.
¶Niamid, 1-2-(benzylcarbamyl ethyl)-2-isonicotinyl-hydrazine, Pfizer Laboratories.

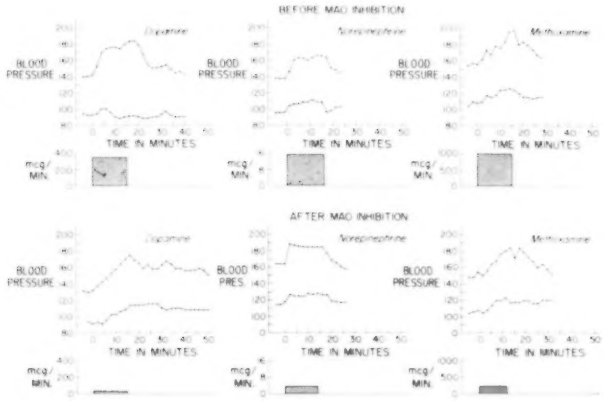

Fig. 1.—Effects of intravenous infusions of dopamine, norepinephrine, and methoxamine before and during therapy with JB-516 in patient G. W. S.

symptoms. During treatment with JB-516 and SKF-385, marked potentiation of the pressor action of dopamine was observed in all patients so that 25 mm. Hg increments in systolic pressure were now produced by infusions of only 5 to 11 per cent of the control doses. There was also pronounced prolongation of the effects of the dopamine infusion, blood pressure elevations lasting as long as 60 minutes, compared with 4 to 9 minutes before inhibitor therapy. Additionally, during therapy, the diastolic pressure frequently became elevated, and the pulse rate decreased as much as 8 beats per minute. Typical responses are shown in Fig. 1.

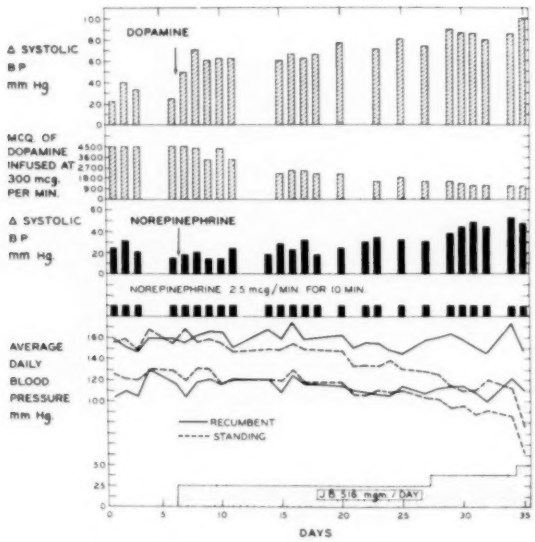

Fig. 2.—The pressor responses to short infusions of norepinephrine and dopamine and the development of postural hypotension in subject J. O. N. while receiving the MAO inhibitor, JB-516. (See text for details.)

In control studies, norepinephrine and methoxamine produced increments in systolic and diastolic blood pressures as well as bradycardia of from 5 to 22 beats per minute. During therapy with JB-516 or SKF-385 the pressor effects of these amines were also potentiated (Fig. 1) though to a lesser extent than with dopamine and without prolongation of action. As may be seen in Table I, the dose of norepinephrine required to produce a 25 mm. increment in systolic pressure was reduced to 13 to 38 per cent of the control dose and that of methoxamine was similarly reduced to 30 to 39 per cent. The bradycardia produced by these amines was decreased in three patients and remained unchanged in the others.

Because of the differences between the marked and prolonged potentiation of the pressor effects of dopamine by the MAO inhibitors and the less marked augmentation of the effects of norepinephrine and methoxamine, two additional studies were performed. First, nialamide, a potent MAO inhibitor reported to have minimal hypotensive actions,[7] was administered to three patients in a dosage of 200 mg. per day. The MAO inhibition produced was equivalent to that previously obtained with JB-516 and SKF-385 as determined by tryptamine excretion. In one patient (A. F.), moderate postural hypotension developed, but hypotension did not occur in the others (W. C. and G. W. S.). The pressor effect of dopamine was potentiated in each of the patients, but augmentation of the actions of methoxamine and norepinephrine occurred only in the patient with postural hypotension. In this patient, greater amine potentiation and more pronounced postural hypotension occurred when the dose of nialamide was increased to 400 mg. per day.

The second additional study consisted of administering daily infusions of dopamine at rates of 300 mcg. per minute and norepinephrine at rates of 2.5 mcg. per minute to patient J. O. N., starting one week before and continuing during therapy with JB-516 at an initial dose of 25 mg. per day (see Fig. 2). Infusions of these amines for 15 minute periods before therapy resulted in average systolic pressure increments of about 30 mm. Hg. An augmented pressor response to the dopamine infusion was apparent after one day of therapy and the potentiation increased progressively. At the end of 2½ weeks of therapy, the systolic pressure rose 70 mm. Hg after 7 minutes of the dopamine infusion. Upon increasing the dose of JB-516 to 37.5 mg. per day the response increased further and a 100 mm. Hg increment in systolic pressure was obtained with only 4 minutes of infusion on the twenty-ninth day of therapy. In contrast, definitive augmentation of the pressor response to norepinephrine did not take place until the fourth week of therapy when pronounced postural hypotension occurred for the first time. At this point the 15 minute infusion of norepinephrine produced a 50 mm. increment in systolic pressure.

DISCUSSION

This is the first demonstration of the pressor effect of dopamine in man. Previous studies in animals have shown that dopamine is pressor in the cat, depressor in the rabbit and guinea pig,[8] and has a variable but predominantly

pressor effect in the dog.[9] The effects of intravenous infusions of dopamine in man were significantly different from those of norepinephrine, for systolic pressure was predominantly elevated by dopamine, whereas norepinephrine increased systolic and diastolic pressure. Such results suggest that dopamine produces an elevation in blood pressure by increasing cardiac output rather than by causing peripheral vasoconstriction. Preliminary physiologic studies, which demonstrated marked increments in cardiac output with decrements in peripheral resistance, have confirmed this impression.

The pronounced augmentation and prolongation of the pressor effects of dopamine in man after MAO inhibition is in agreement with reports on the potentiation of the cardiovascular actions of this amine in animals by a number of inhibitors.[9, 10] Potentiation of dopamine could be seen in the absence of postural hypotension and seemed to bear a direct relationship to the degree of MAO inhibition produced as measured by urinary tryptamine levels. This suggests that the augmentation of the pressor effects of dopamine may be used as a sensitive indicator of MAO inhibition in man. Since dopamine was found to be a pressor rather than a depressor agent in man, a role for this amine in the production of postural hypotension by MAO inhibitors is not apparent. However, a similar paradox exists in patients with excess norepinephrine due to pheochromocytoma who exhibit postural hypotension.[11] It is possible that excessive tissue levels of dopamine and other endogenous amines may result in sympathetic blockade.

The potentiation of the pressor effects of norepinephrine in the present study was unexpected in view of previous reports of the lack of augmentation of the cardiovascular actions of this amine in animals treated acutely with inhibitors[9] and in patients treated with iproniazid.[12, 13] It should be noted that augmentation of norepinephrine in the present study occurred only after the development of postural hypotension. That it did not appear to be directly related to MAO inhibition is suggested by the following: (1) pronounced MAO inhibition could be established as determined by tryptamine excretion without altering the effects of norepinephrine; (2) when the pressor effects of norepinephrine were augmented there was no prolongation of effects as seen with dopamine, and (3) the pressor effects of methoxamine, which is not metabolized by MAO, were similarly augmented after development of postural hypotension. Since methoxamine has no direct cardiac effects in man[14] but raises blood pressure only by peripheral vasoconstriction, such augmentation must be due to enhanced peripheral sensitivity.

The present results do not establish the basis for augmentation of the effects of norepinephrine and methoxamine but suggest that it is correlated with the production of postural hypotension by the MAO inhibitors. The site of action of the inhibitors in producing postural hypotension has not yet been proved, although it appears to be the result of sympathetic block[1, 15] perhaps in the sympathetic ganglia.[16] It is well known that the pressor effects of norepinephrine are augmented by conventional ganglionic blocking agents.[17]

CONCLUSIONS

Intravenous infusions of dopamine increased systolic blood pressure in seven hypertensive patients without altering the diastolic pressure. During therapy with three potent monoamine oxidase inhibitors, JB-516, SKF-385, and nialamide, the pressor effects of dopamine were markedly potentiated and prolonged. This potentiation appeared to be the result of monoamine oxidase inhibition. The pressor effects of intravenous infusions of norepinephrine and methoxamine were also augmented during therapy with the monoamine oxidase inhibitors, but to a lesser extent and without prolongation of effects. Augmentation of the actions of the latter amines was correlated with the development of postural hypotension and did not appear directly related to monoamine oxidase inhibition.

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THE ARGINASE ACTIVITY OF HUMAN LEUKOCYTES

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Because of the controversial nature of the few reports available in the literature, human leukocyte arginase activity has been studied by an improved spectrophotometric method in a variety of hematologic and nonhematologic diseases. The mean leukocyte arginase level (expressed per 10^{10} white blood cells) in chronic granulocytic leukemia was found to be above the mean of normal individuals, but quite similar to the mean in several disease categories. When expressed in terms of 10^{10} neutrophils, the mean in chronic granulocytic leukemia was not elevated. There is suggestive evidence that the leukocytes in the megaloblastic anemias and in the severe forms of thalassemia share an increased level of arginase activity similar to that of the erythrocyte in these diseases. Further data are presented which confirm previous reports of little, if any, arginase activity in lymphocytes and blast cells of all types. Mature neutrophils appear to have the greatest degree of activity on a per cell basis. It is concluded that normal and diseased leukocytes possess variable amounts of arginase activity.

THE arginase activity of human erythrocytes and leukocytes with particular reference to the consistently elevated erythrocyte arginase activity in megaloblastic anemias in relapse and in thalassemia major was previously reported from this laboratory.[1] It was noted that leukocytes possess arginase activity, but no distinctive pattern of leukocyte arginase values in various diseases affecting white blood cells was found, except that in chronic lymphocytic leukemia, infectious mononucleosis, acute leukemia, and the terminal blastic phase of chronic granulocytic leukemia, leukocyte arginase values were lower than normal. From these findings it was inferred that granulocytes, as opposed to mononuclear or blast cells, contribute primarily to the total arginase activity of leukocytes.

No arginase activity, however, was found in human white blood cells by Kochakian and co-workers.[2] On the other hand, Borghetti and Scarpioni[3] have reported increased leukocyte arginase values in chronic granulocytic leukemia. Because of the paucity and controversial nature of the data available in the literature in regard to leukocyte arginase activity, we have extended our arginase studies with emphasis on the activity of this enzyme in separated human leukocytes. These data, together with significant improvements in the procedure which make the determination of arginase feasible even in the presence of leukopenia, form the basis of this report.

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MATERIALS AND METHODS

Leukocytes were separated from the erythrocytes of freshly collected human venous blood by the use of a 4 per cent solution of bovine fibrinogen (Sigma) in physiologic saline. A saline suspension of leukocytes in a concentration range of 30,000 to 40,000 cells per cubic millimeter was prepared as previously described.[1] An erythrocyte contamination of about 50,000 cells per cubic millimeter was considered insignificant insofar as no detectable arginase activity could be demonstrated on repeated arginase determinations in this range of pure erythrocyte concentration. If the erythrocyte contamination were greater, the arginase activity of the erythrocytes was determined simultaneously and appropriate correction was applied. Arginase activity was determined by the method previously described,[1] except for the following modifications:

1. Two tenths milliliter of a white blood cell suspension in normal saline was added to each incubation pool instead of the previous 1.0 ml. The volume of stock standard urea solution and distilled water was reduced to 0.2 ml. per tube. For the determination of erythrocyte arginase activity, the volume of hemolyzed red cells was also reduced to 0.2 ml.

2. The incubation time at 37° C. was reduced from 6 to 3 hours.

3. The final reading (taken immediately after adding Nessler's reagent) was performed on a Beckman model B spectrophotometer with a blue phototube at a wavelength of 410 mμ instead of the former 540 mμ.

In experiments in which ammonium chloride or sulfate was used, we have found that a wavelength of approximately 410 mμ is optimal for the conditions of our method of arginase determination. Hence, all of our data have been collected at this wavelength. This greatly increased the sensitivity of the test and markedly decreased the number of blood cells required to give absorbencies in the optimal photometric range. While our studies were in progress, Henry and Chiamori[4] reported that reading at approximately 420 mμ (in contrast to the 480 to 540 mμ range commonly employed) increases the accuracy and sensitivity when direct nesslerization of ammonia formed by urease treatment of whole blood, serum, and urine is employed.

The values for arginase activity are expressed as milligrams of urea nitrogen liberated per hour from L-arginine substrate at 37° C. by 10^{10} white blood cells or by 10^{10} neutrophils. In this article, the term neutrophil will refer to myelocytes, metamyelocytes, bands, and segmented neutrophils inclusively. The 200 cell differential counts were performed on peripheral smears obtained at the time the blood samples were obtained for analysis.

RESULTS

The pertinent data of all the disease categories studied for arginase activity of leukocytes are summarized in Table I, and the individual values (activity expressed per 10^{10} white blood cells) are presented in Fig. 1 in the form of a scattergram. The mean value for each group expressed in terms of 10^{10} neutrophils is shown in Fig. 2 in the form of a bar graph.

One patient with chronic granulocytic leukemia (not included in the tabulated series) had an arginase value of 260 per 10^{10} white blood cells in the presence of 78.5 per cent basophils. Seven determinations on 4 patients with chronic granulocytic leukemia who were studied during an acute exacerbation of their disease revealed a mean of 34 per 10^{10} white blood cells. In general, the values decreased as the percentage of blasts increased. This pattern was also seen in the patients with acute leukemia. During intervals of partial remission when the percentage of neutrophils increased, there was a corresponding increase in the arginase activity when expressed in terms of 10^{10} white blood cells.

TABLE I

NO. PATIENTS STUDIED	DIAGNOSIS	MEAN W.B.C.	MEAN % NEUTROPHILS*	MEAN ARGINASE† PER 10^{10} W.B.C.	MEAN ARGINASE† PER 10^{10} NEUTROPHILS	RANGE OF ARGINASE† PER 10^{10} NEUTROPHILS
9	Normal (7 men, 2 women; age range 20-58)	7,533	59.8	105	195	111-239‡
16	Chronic granulocytic leukemia	133,258	71.6	130	184	73-357
30	Acute leukemia (all types; mostly adults)	68,057	13.1	36	—§	—§
17	Chronic lymphocytic leukemia	116,495	8.0	22	—§	—§
19	Infectious mononucleosis	9,778	31.5	73	231	32-377‖
7	Myeloid metaplasia	63,505	78.4	137	175	65-316
9	Polycythemia rubra vera	26,411	79.3	164	209	91-276
6	Thalassemia minor	9,433	56.7	145	278	182-419
9	Pernicious anemia	5,373	50.4	133	269	142-570
8	Cirrhosis of the liver	10,225	67.4	132	204	129-347
22	Miscellaneous hematologic diseases	12,207	65.7	127	201	75-406
46	Miscellaneous nonhematologic diseases	11,571	67.3	119	177	56-354
14	Neutrophilic leukocytoses¶	18,180	89.9	175	195	93-322

Mean lymphocyte per cent in chronic lymphocytic leukemia = 89.9; in infectious mononucleosis = 63.2.

*Myelocytes to segmented neutrophils included.

†Expressed as milligrams of urea nitrogen liberated per hour.

‡One value of 305 in normal subject with only 40 per cent neutrophils excluded.

§Not calculated due to low percentage of neutrophils.

‖Single value of 495 excluded.

¶Includes miscellaneous diseases with greater than 84 per cent neutrophils.

In patients with infectious mononucleosis, the mean value of 231 when expressed in terms of 10^{10} neutrophils may be erroneously elevated because of the low percentage of neutrophils. Serial determinations in one patient revealed values of 36, 33, 69, 148, and 175, per 10^{10} white blood cells. These values correlated well with an increasing percentage of neutrophils during recovery.

The thalassemia minor group is actually composed of 4 patients with thalassemia minor and 2 individuals with the intermediate form. The latter 2 patients had the highest values in the group (363 and 419 per 10^{10} neutrophils).

Included in the pernicious anemia category were 8 patients with pernicious anemia and 1 patient with postgastrectomy megaloblastic anemia. All except one of these patients had elevated erythrocyte arginase values. Weekly assays for a period of 6 weeks in one patient and serial determinations (10) in another patient revealed a gradual decrease in leukocyte (as well as erythrocyte) arginase activity to normal levels after institution of vitamin B_{12} therapy. One patient with megaloblastic anemia due to folic acid deficiency (not included in tabulated series) had a value of 312 per 10^{10} white blood cells or 345 per 10^{10} neutrophils.

DISCUSSION

It is apparent from the foregoing results and from the data presented previously[1] that human leukocytes have variable amounts of arginase activity in health and in disease states. It is also quite apparent that the various cell types in the peripheral blood contain different amounts of arginase activity. Our

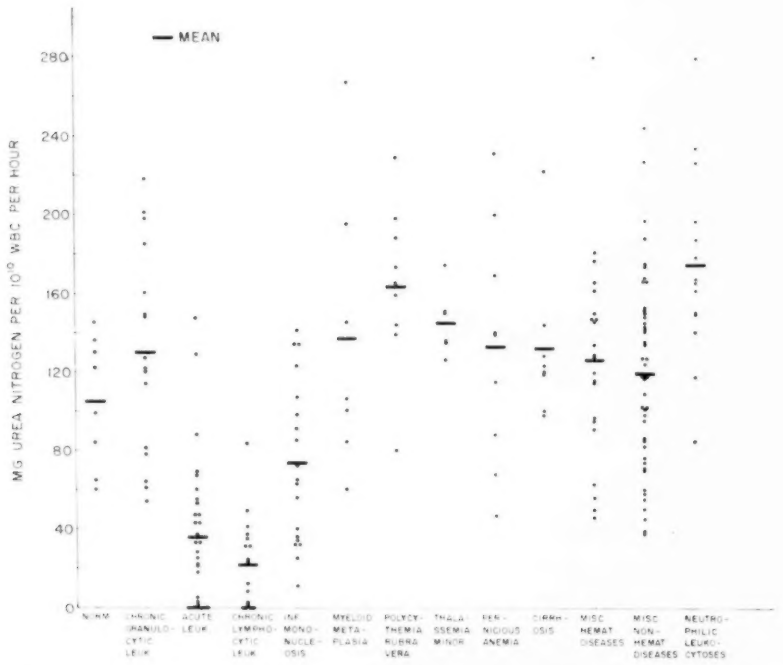

Fig. 1.—Scattergram of arginase activity of leukocytes obtained from patients with various diseases. Each dot represents a different patient, except in the extreme right column, which is composed of those patients with greater than 84 per cent neutrophils in the miscellaneous diseases groups. Values for arginase activity are expressed as milligrams of urea nitrogen liberated per 10^{10} white blood cells per hour at 37° C.

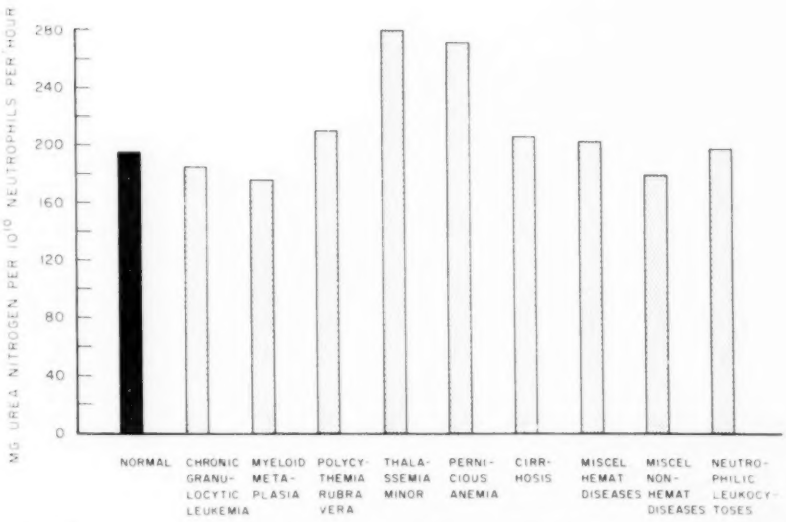

Fig. 2.—The mean values (arginase activity expressed in terms of 10^{10} neutrophils) for normal subjects and for the disease categories are shown. Note that the mean values are quite similar except for thalassemia minor and pernicious anemia.

present results confirm our previous report[1] that leukocyte arginase values when expressed in terms of 10^{10} white blood cells are lower than normal in acute leukemia of various types, chronic lymphocytic leukemia, infectious mononucleosis, and the blastic phase of chronic granulocytic leukemia (Fig. 1). However, when expressed in terms of 10^{10} neutrophils (as defined earlier in this paper), the arginase values in these diseases are essentially normal. From these findings, one may reasonably infer that lymphocytes and blast cells of all types contain minimal, if any, arginase activity and that the neutrophils probably contribute most of the measurable arginase activity of human leukocytes. Borghetti and Scarpioni[3] have noted that patients with chronic lymphocytic leukemia (in the presence of 99 per cent lymphocytes) have no arginase activity in their leukocytes and that there is diminished arginase activity in leukocyte suspensions with a low percentage of cells of the granulocytic series. From these observations, they have concluded that there is probably no arginase activity in lymphocytes.

Borghetti and Scarpioni have also reported that there is an increase in leukocyte arginase activity in patients with chronic granulocytic leukemia as compared to normal subjects. They have suggested that the increase of arginase activity in leukemic granulocytes may be a reflection of altered cellular metabolism. The mean of our arginase results (expressed per 10^{10} white blood cells) in chronic granulocytic leukemia is higher than our normal mean, and thus our results would appear to agree with those of Borghetti and Scarpioni. However, Fig. 1 demonstrates that the range and mean of chronic granulocytic leukemia are quite similar to most of the disease categories studied, except for the groups already mentioned (vide supra). Furthermore, when the arginase results are expressed in terms of 10^{10} neutrophils, the mean in patients with chronic granulocytic leukemia is comparable to the mean in our normal subjects (as well as to the mean of most of the disease categories studied, except for thalassemia and pernicious anemia) (Fig. 2). It would thus appear that the elevation of arginase activity (expressed per 10^{10} white blood cells) in patients with chronic granulocytic leukemia as compared to normal subjects may be largely a reflection of the increased percentage of neutrophils. It is also to be noted that the mean values in chronic granulocytic leukemia, whether expressed in terms of 10^{10} white blood cells or 10^{10} neutrophils, are very similar to those in myeloid metaplasia, but are slightly lower than those in polycythemia rubra vera or miscellaneous diseases with a high percentage of neutrophils (Table I). In addition, the arginase activity has been noted to decrease during acute exacerbations of chronic granulocytic leukemia. These observations, considered together with those already cited, are consistent with the concept that mature neutrophils probably are the principal contributors to total leukocyte arginase activity in the usual white blood cell population. It is also apparent that the assay for leukocyte arginase activity is of no differential diagnostic aid in attempting to differentiate one from another in the myeloproliferative group of diseases. Our results in the chronic granulocytic leukemia group indicate that basophils possess arginase activity, but there was no consistent relationship between the level of arginase activity and percentage of basophils.

It has been established that the erythrocytes of patients with megaloblastic anemia in relapse and of patients with thalassemia major have elevated arginase activity.[1, 2] Although our series was small, it is interesting to note that the mean leukocyte arginase activity per 10^{10} neutrophils was the highest in these two disease groups (Fig. 2), though the wide range of activity in all disease groups is, of course, recognized. The highest leukocyte arginase activity among the patients with megaloblastic anemia occurred in the patient with the highest erythrocyte arginase level and serial assays in two patients have indicated that the elevated leukocyte arginase levels on a 10^{10} neutrophilic cell basis return to normal levels after therapy. In addition, the two patients with the intermediate form of thalassemia had higher leukocyte arginase values than did the patients with the diagnosis of thalassemia minor. Although all of these observations are limited in extent, it appears that the leukocytes of patients with megaloblastic anemia and probably the more severe forms of thalassemia share an increased level of arginase activity previously demonstrated in the erythrocyte in these diseases.

It has been reported that the erythrocyte arginase activity of patients with chronic liver disease decreases remarkably.[3] However, our small group of patients with cirrhosis of the liver had normal erythrocyte arginase levels and the leukocyte arginase activity was also normal.

SUMMARY

Human leukocyte arginase activity has been studied in a variety of hematologic and nonhematologic diseases. Significant improvements in our assay method have been presented.

A wide range of leukocyte arginase activity exists in almost every disease category. The mean values, however, when expressed in terms of 10^{10} neutrophils are quite similar in the diverse disease categories studied with the possible exception of the megaloblastic anemias and the severe forms of thalassemia. There appears to be no pathognomonic disease pattern for leukocyte arginase activity.

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INFLUENCE OF SITE OF IMPLANTATION ON LIPID CONTENT OF SUBCUTANEOUS SPONGE BIOPSY CONNECTIVE TISSUE

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Polyvinyl surgical sponges were implanted subcutaneously in various positions in the dorsa of adult rats and were examined microscopically and chemically after various time intervals. The extractable lipid content of the new connective tissue in the sponge biopsies was found to vary depending upon whether the sponge implantation site adjoined an adipose tissue depot or whether it was situated in a leaner area of the mid-back. Microscopically, most fat seemed to arise from pre-existing adipose tissue. It is shown that the reporting of chemical values for nonlipid tissue components such as hexosamine and collagen on a dry (lipid containing) basis can be misleading, especially when large variations in lipid contents are present.

THE sponge biopsy technique of Boucek and Noble[1] has added a welcome tool for the study of connective tissue. Polyvinyl sponges, implanted subcutaneously, become invaded by connective tissue and can be harvested at various intervals for chemical or microscopic examination. Dunphy and associates[2] have pointed out that the rate of growth and chemical composition of new tissues vary, depending upon whether sponges are implanted in muscle or in subcutaneous tissue. More recently it has been shown that new bone may infiltrate plastic sponges when the latter are implanted in properly prepared troughs made in femurs of adult rats.[3] Studies in our laboratories indicate that composition of sponge biopsy tissue may vary with site of implantation even when all sponges are implanted subcutaneously. Presence or absence of juxtaposed fat appears to be especially influential.

METHODS AND MATERIALS

Male albino rats of the Sprague-Dawley strain and 3 to 4 months of age were used. All rats were fed finely milled Rockland rat stock diet.

For chemical examination, sheets of polyvinyl surgical sponge (Ivalon), 3 to 4 mm. in thickness, were cut into circles 13 mm. in diameter by means of a No. 8 cork borer. These sponges had a washed, dried weight of 20 ± 2.2 mg. Those intended for microscopic study were square (10 by 10 by 3 to 4 mm.). All sponges were prepared for implantation by washing in running tap water for 18 hours followed by thorough rinsing in distilled water. They were dried in a vacuum oven, weighed, and sterilized by autoclaving in distilled water for 20 minutes. Normal saline was not used to moisten the sponges since

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it appears that more uniform responses are obtained when sponges are moistened with distilled water.[4] Wet sponges were implanted aseptically in rats under combined pentobarbital sodium and ether anesthesia through a longitudinal incision made near the midline of the dorsum. They were implanted in subcutaneous pockets in various positions, care being taken to place all sponges at least 1.0 cm. away from the incision and lateral to the midline. Positions varied from the adipose tissue running dorsoventrally in the scapular region, through the leaner area of the mid-back, to the dorsoventral fatty area in the hip region. In one rat, 2 sponges were placed intraperitoneally, one being anchored to the omentum by means of a suture, the other being left free. The latter, after 7½ weeks, was found imbedded in extraperitoneal inguinal fat.

For microscopic examination, sponges were excised together with some of the surrounding tissue. They were fixed in neutral formalin, embedded in paraffin, sectioned, and stained. For chemical examination, sponges were stripped of their surrounding capsule as cleanly as possible. Moisture was determined by drying in a vacuum oven at 50° C. for 4 hours. Lipid was determined by extracting the dried, unground tissue for 4 to 5 hours with each of successive 6 ml. portions of 3:1 ethanol-ether (2 times), petroleum ether, and 3:1 ethanol-ether. Collagen extracts of moisture-free, lipid extracted residues were prepared and their hydroxyproline contents were determined by the methods of Neuman and Logan.[5, 6] Hydroxyproline was converted to collagen by multiplying by 7.46.[5] Hexosamine was determined by the method of Boas.[7] All weights were corrected for content of inert sponge material; all percentages are therefore expressed in terms of sponge-free tissue.

RESULTS AND DISCUSSION

Variation in Sponge Biopsy Lipids.—Table I contains data illustrating the variation that may occur in the lipid contents of sponges implanted subcutaneously in the same animal (note especially Rats 40, 44, and 46). With one exception, where 2 sponges were implanted on the same side of the animal, the sponge placed closer to the subscapular fat deposit had a higher lipid content. In the one exception, the sponge with the higher lipid content adjoined the fat deposit near the hip, the other being located in the mid-back (Table I, Rat 40). That nutritional status of the animal may also influence fat content of biopsy tissue seems to be indicated by results from Rat 48, which lost 100 grams in weight during the last half of the experimental period because of restricted food consumption.

Microscopic examination of sponge biopsies revealed that those areas adjoining fat depots had a much higher fat content than areas not in contact with fatty tissue (Figs. 1, 2, and 3). In general, there was more fat near the periphery of the sponges than in the center. Our studies have given us the impression that fat cells within sponges were always contiguous to other fat cells. No typical, immature fat cells with numerous fat droplets in the ectoplasm have been observed. This seems to indicate that fat within the sponge is formed largely from pre-existing adipose tissue and that few if any fat cells arise from other types of cells within the sponge.

It appears obvious that care must be taken in selecting comparable sites for subcutaneous sponge implantations. Our observations indicate that the number of sponges that can be implanted in comparable sites in a single adult rat is strictly limited to a relatively small area in the mid-back.

Expression of Analytical Results.—In reporting levels of nonlipid components determined by tissue analysis, selection of a logical basis of expression

TABLE I. LIPID CONTENT OF SPONGE BIOPSY TISSUE

RAT NO.	AGE OF IMPLANT (DAYS)	WET WEIGHT OF TISSUE (MG.)	H_2O (%)	LIPID (%)	RESIDUE (%)
40	30*	271.0	74.0	15.7	10.3
	30	243.8	82.0	1.6	16.4
41	30†	227.0	83.0	4.7	12.3
	30	260.5	86.4	0.5	13.1
	30	180.3	83.5	1.8	14.7
44	30†	320.7	65.2	24.4	10.4
	30	270.3	82.4	4.1	13.5
45	30†	313.4	80.1	5.2	14.7
	30	312.2	85.0	1.5	13.5
46	30†	343.3	69.3	17.2	13.5
	30	248.4	81.2	4.4	14.4
47	30†	312.5	77.8	8.8	13.4
	30	225.6	82.6	2.7	14.7
42	84†	247.2	79.7	5.5	14.8
	84	236.6	82.9	1.8	15.3
43	84†	337.9	78.4	4.6	17.0
	84	243.0	82.1	2.9	15.0
	84	243.2	83.8	2.0	14.2
48‡	84†	275.5	86.1	0.7	13.2
	84	269.2	86.4	0.1	13.5
	84†	242.3	87.7	0.6	12.7
55	98†	315.2	77.0	8.4	14.6
	98	258.6	80.0	6.3	13.7
59	98†	306.0	78.9	6.1	16.3
	98	336.6	82.8	3.6	13.6
	98†	331.7	77.4	7.9	14.7
	98	268.8	81.3	3.9	14.8

Implantation sites not otherwise specified were in the mid-back area. Where two implantation sites are indicated to be the same in the same rat, they represent comparable sites on opposite sides of the midline.

*Adjoining fat deposit near hip.

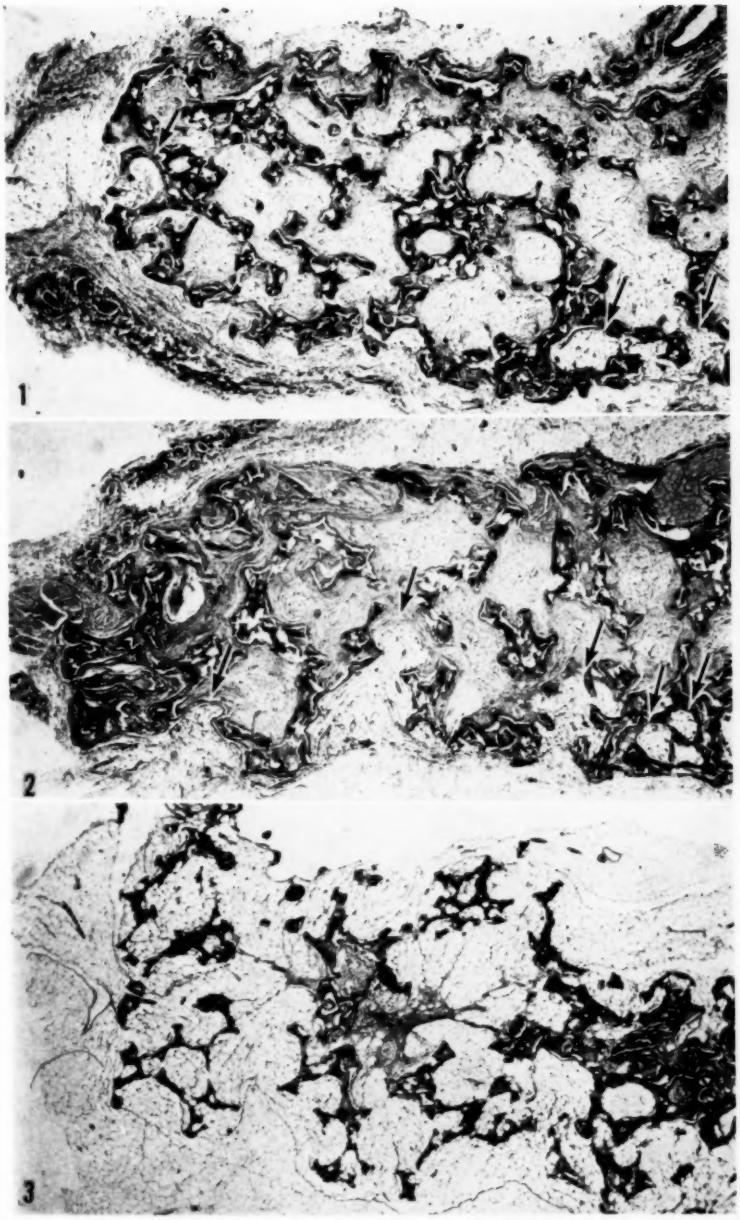
†Toward, or adjoining, subscapular fat deposit.

‡Emaciated, initial and final weights were 361 and 260 grams, respectively.

TABLE II. EFFECT OF MODE OF EXPRESSION IN SPONGE TISSUE ANALYSES

RAT NO.	LIPID CONTENT OF TISSUE (%)		COLLAGEN OR HEXOSAMINE CONTENT OF TISSUE (%)		
	WET BASIS	DRY BASIS	WET BASIS	DRY BASIS	DRY, LIPID EXTRACTED RESIDUE BASIS
				Collagen	
40	15.7	60.4	2.01	7.7	19.5
44	4.1	23.3	2.56	14.5	19.0
45	1.5	10.0	2.76	18.4	20.4
				Hexosamine	
			(mg./100 Gm.)	(mg./100 Gm.)	(mg./100 Gm.)
42	1.8	11.8	76.1	445	487
43	2.0	12.5	90.0	568	640
46	17.2	56.0	95.8	312	710

is frequently a problem. Results may be expressed as per cent of fresh wet tissue, as per cent of dry tissue, or as per cent of moisture-free lipid extracted residue. A commonly used basis of comparison is the dry weight of the tissue. In a series of biopsy specimens such as is represented in Table I, where



Sections through sponges from different implantation sites in the same animal. Implanted 53 days. (Van Gieson stain. Magnification ×30; reduced ½.)

Fig. 1.—Mid-back region. Small areas of adipose tissue within actual sponge area are indicated by arrows.

Fig. 2.—Scapular or subscapular region. Several areas of adipose tissue within sponge area indicated by arrows and located in that part of sponge immediately adjacent to subscapular fat pad.

Fig. 3.—Inguinal region. Almost complete occupation of sponge area by adipose tissue. This sponge was placed free in the peritoneal cavity, but was found embedded in extraperitoneal adipose tissue of the inguinal region when animal was sacrificed. A sponge anchored to greater omentum by a suture showed essentially the same distribution of fat.

per cent of residue is more constant than lipid content, this would give rise to a serious distortion of the true picture for all nonlipid components. A more logical expression would appear to be based on weight of dry, lipid extracted tissue (residue), as recently used by Bollet and co-workers[8] to report analyses of sponge biopsy tissues. Another satisfactory way of expressing sponge biopsy analyses is based on weight of dry sponge implanted.[9]

Table II illustrates the effect of using different bases for expression of collagen and of hexosamine levels when sponge biopsies having different lipid contents are compared. It should be noted that the 3 collagen levels are about the same when calculated on the basis of dry, lipid extracted residue. Yet, when calculated on the basis of dry (lipid containing) tissue, the highest value is almost 2½ times that of the lowest. In the case of the tabulated hexosamine levels (Table II), the highest level, based either on fresh tissue weight or on residue weight, becomes the lowest value when recalculated on the basis of dry tissue weight. Use of dry tissue weight in these instances actually misrepresents true hexosamine and collagen levels of tissues because of the "diluting" effect of lipid in the calculations.

SUMMARY

1. Connective tissue specimens obtained from polyvinyl sponges implanted subcutaneously in adult rats varied greatly in extractable lipid content depending upon the site of sponge implantation. Sponges adjoining adipose tissue depots contained markedly increased levels of lipid when compared to sponges implanted in lean areas of the midback. Lipid content also appeared to be influenced by the nutritional status of the animal.

2. Microscopically, all fat cells in sponge biopsy tissue appeared contiguous to other fat cells. It seems likely that most if not all fat in the biopsy arose from pre-existing adipose tissue.

3. The effect of using different bases for expression of sponge biopsy analyses is illustrated and discussed. It is shown that the reporting of values for nonlipid components on a dry (lipid containing) tissue basis can be misleading, especially when large variations in lipid contents are present.

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LABORATORY METHODS

COLORIMETRIC DETERMINATION OF BLOOD AMMONIA

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Determination of blood ammonia is performed in two steps. Satisfactory methods are available for the first step which is the separation of ammonia from blood by microdiffusion. Other problems such as the rapid increase of ammonia in blood samples are troublesome. The time between venipuncture and analysis may be extended to 30 minutes when the samples are iced. The colorimetric determination of ammonia with sodium phenolate and hypochlorite, catalyzed by nitroprusside, is more sensitive than the ninhydrin method and is more reproducible and convenient than the Nessler method. Excellent precision is obtained in the range 0.5 to 6.0 mcg. of ammonia.

Data are presented regarding the effect of time and temperature, hemolysis, differences in arterial and venous blood ammonia, and differences between diffusion and recovery of ammonia from blood and water.

The concentration of blood ammonia depends on the conditions and methods of measurement, but fairly precise limits for normal and abnormal can be established if the various factors are controlled carefully.

THE quantitative estimation of micro quantities of ammonia in blood is performed in two steps: the separation of the ammonia from the blood by microdiffusion and the measurement of the ammonia by titrimetric or colorimetric procedures.

The colorimetric reaction between NH_3 and sodium phenolate in the presence of hypochlorite yields a stable blue dye when catalyzed by sodium nitroprusside. Brown and coauthors[1] reported the use of this reaction for analysis of plasma and tissue ammonia. In our experience the sodium phenolate reaction offers many advantages also for the determination of ammonia in blood. In addition, we have devised a microdiffusion method of separation of the ammonia from the blood which has been convenient and reproducible.

METHODS AND MATERIAL

The diffusion equipment is simple and consists of (1) Warburg flasks without center wells and fitted with No. 0 black rubber one holed stoppers through which a 3 cm. piece

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of glass tubing (Pyrex) sealed at one end has been inserted so the open end is in the flask, (2) a shaker with a to and fro motion and variable speeds, and (3) an insulated chest for temperature control. The colorimetric measurement is performed with a Beckman DU spectrophotometer or the Coleman Junior spectrophotometer or suitable colorimeter.

The reagents and their preparations are as follows:

1. *A saturated solution of sodium carbonate* prepared by adding 50 parts of sodium carbonate to 100 parts of double distilled water, heated to boiling for 10 minutes. A few crystals will not dissolve. After cooling, a crystal-free solution is obtained by decantation into a clean Pyrex bottle. Potassium carbonate may be used instead of sodium carbonate.

2. *Solution (0.05M) of sodium carbonate.*

3. *Sodium nitroprusside solution* (0.5 per cent). A 1:100 dilution of this solution is made up fresh for each day's analyses.

4. *Solution of sodium phenolate* (2.5 per cent). Five grams of reagent grade phenol and 2.5 Gm. of reagent grade sodium hydroxide are added to 200 ml. of ammonia-free water. This solution must stand 48 hours before it is used.

5. *Sodium hypochlorite solution* (about 0.06N). This solution is prepared by diluting a stock solution of sodium hypochlorite (5 to 6 per cent available chlorine) 1:20. Sodium thiosulfate titration is used for the standardization of this solution to verify that it is in the range of 0.05 to 0.07N.

6. *Sulfuric acid* (1N).

7. *Ammonia-free* water, prepared by distillation from acid, is used in the preparation of all the solutions except that of the saturated sodium carbonate. Solutions are not stored for longer than a week because the blank readings slowly increase. The optical density of the blanks with fresh reagents is less than 0.050.

Contamination of reagents or glassware by ammonia vapor must be avoided. Syringes, needles, test tubes, stoppers, and glassware are cleaned with acid and rinsed in ammonia-free water. Ammoniacal solutions or urine are not allowed in the room where ammonia analyses are performed. Glassware is kept in covered containers.

Blood for analysis is drawn with the specially cleaned heparinized syringes and needles. The blood is drawn without the use of a tourniquet. Hemolysis is avoided. No bubbles are permitted. The sample tubes are stoppered and iced immediately and analysis is begun in less than 30 minutes after venipuncture.

Separation of Ammonia From Blood by Micro Diffusion.—The diffusion process is standardized and kept constant from experiment to experiment. Variations in temperature, shaking speed, or diffusion surface (size of flask relative to volume of sample) are not permitted because they affect diffusion and the recovery of ammonia. The volumes of standard and unknowns should be the same and in these experiments, 1.0 ml. was used. Unless otherwise indicated, the diffusion time was 30 minutes.

With every experiment, a standard that contains a known quantity of ammonia in water is diffused and the recovery is compared with previous experiments in order to verify that the diffusion conditions are the same.

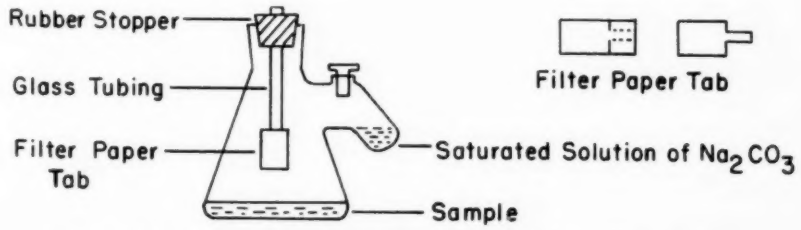

Fig. 1.—Warburg flask used as diffusion vessel for ammonia analysis.

All analyses are made in triplicate. The reaction vessels (Warburg flask) are set up as illustrated in Fig. 1. Add 1.0 ml. of saturated solution of sodium carbonate to the side arm. Place 1.0 ml. of H_2O, standard, or blood, in the bottom of the flask. Insert the filter paper tab in the open end of the glass tubing and moisten it with 1N sulfuric acid and stopper the flask. Start the diffusion process by tipping the side arms so the saturated sodium carbonate mixes with the sample. Place the flasks on the shaker. During diffusion the ammonia is collected on the filter paper tab. After 30 minutes, remove the flasks from the shaker and immediately drop the filter paper tabs into separate test tubes. The time the flasks are shaken is called the "diffusion time."

Colorimetric Measurement of Ammonia.—One milliliter of each of the following solutions is then added to the test tube in the following order: (1) ammonia-free water, (2) sodium phenolate, 2.5 per cent solution, (3) 0.5 per cent sodium nitroprusside solution diluted 1:100, (4) 0.05 molar sodium carbonate solution, and (5) sodium hypochlorite solution (about 0.06N). Mix the tubes thoroughly by shaking and allow to stand for 30 minutes at room temperature in order to permit maximal development of color. The color is stable for at least 24 hours (Fig. 2), so that readings can be made at any convenient time after maximal color has developed. Add 5 ml. of distilled water to each test tube to give a final volume of 10 ml. Optical density is read at 625 mμ with a Beckman model DU spectrophotometer or suitable colorimeter. The blood ammonia in the sample is calculated by comparison with the diffused ammonia standard. Blanks and standards which have not been diffused are also analyzed as a check on the colorimetric procedure.

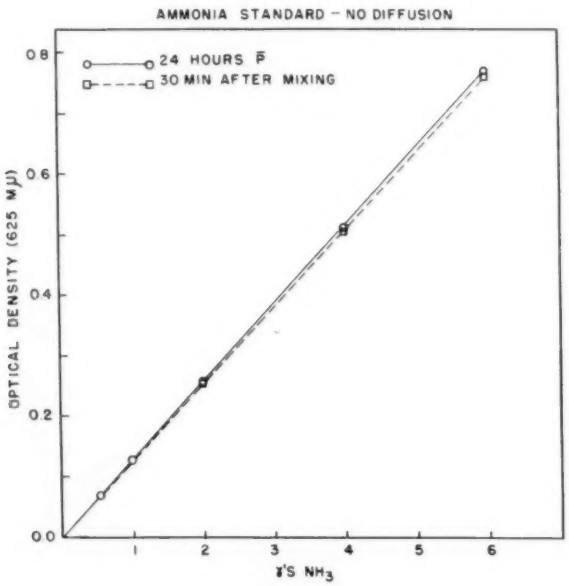

Fig. 2.—Optical density of ammonia standards after reaction with sodium phenolate and hypochlorite. Data from Table I. The solid line is the optical density 24 hours after the reaction.

It is essential that each analysis be made under identical conditions. Factors, such as the lapsed time, the effect of alkali, the different diffusion from blood and water are then identical in all experiments. Comparison of blood ammonia of different samples, different individuals, or different experiments does not require correction for these factors.

RESULTS

The colorimetric procedure is sensitive and reproducible as indicated by data in Table I that show the analysis of ammonia standards. Analyses are done in triplicate and the variance is similar for the six experiments shown. Optical density is proportional to the concentration of ammonia throughout the range of values observed. The optical density of blanks with no added ammonia is only ¼ to ½ the optical density of samples that contained 0.5 meg. of ammonia. The reproducibility of the method is satisfactory even at these low levels. The molecular extinction coefficient (E = 21,000) is at least ten times larger than the Nessler method. The optical density is unchanged after 24 hours at room temperature and the colored product of the reaction is soluble and stable. The colored products of the reaction of ammonia with ninhydrin reagent are also stable, but this method is somewhat less sensitive (E = 15,500).[2]

TABLE I. REPRODUCIBILITY OF COLORIMETRIC METHOD

γ NH_3 PER ML. SAMPLE	EXPERIMENT (OPTICAL DENSITY AT 625 Mμ)						ARITHMETIC MEAN	STANDARD DEVIATION	COEFFICIENT OF VARIATION (%)
	1	2	3	4	5	6			
0.5	0.053	0.061	0.064	0.068	0.067	0.066	0.063	0.006	9.5
1.0	0.117	0.127	0.120	0.119	0.121	0.121	0.121	0.003	2.5
2.0	0.243	0.244	0.246	0.242	0.242	0.250	0.244	0.003	1.2
4.0	0.483	0.493	0.494	0.498	0.489	0.498	0.492	0.006	1.2
6.0	0.757	0.759	0.761	0.742	0.752	0.753	0.758	0.008	1.1

Ammonia standards analyzed without diffusion.
Each value is the mean of triplicate samples. Each column is a different experiment with new reagents.
Data are plotted as Fig. 1.
Molecular extinction coefficient = $O.D._{625}/M./L./cm.$ = 21,000.

The diffusion process which is described separates the ammonia from water or blood in a rapid reproducible manner. The diffusion takes place in a closed vessel after the alkali is tipped in from the sidearm. Data for diffusion and recovery of ammonia from water are shown in Table II. Complete recovery requires long diffusion, but the colorimetric method is so sensitive that complete recovery is unnecessary.

The diffusion and recovery of ammonia from blood are shown in Table III. The triplicate analyses of blood and water are in agreement; the standard deviation in the six experiments likewise shows satisfactory reproducibility of diffusion and recovery. Diffusion of ammonia from blood is less rapid than from water. Recovery is less complete with higher concentrations of ammonia. Similar differences in diffusion and recovery from water and blood have also been noted when other techniques are employed.[3] By our method, 30 minutes' diffusion gives satisfactory recovery from blood.

During the process of diffusion, the alkali which is added to make the ammonia diffusible may split certain labile amides. Under such circumstances, more rapid diffusion would be useful and doubtless readily accomplished by increasing the shaking speed.

TABLE II. EFFECT OF TIME OF DIFFUSION ON RECOVERY OF AMMONIA FROM WATER

DIFFUSION TIME* (MIN.)	NH_3 ADDED (MCG.)	NH_3 RECOVERED (MCG.)	STANDARD DEVIATION	% RECOVERY
15	1.00	0.17	0.08	17
	2.00	0.72	0.15	36
	4.00	1.68	0.08	42
	6.00	2.69	0.22	45
30	1.00	0.63	0.16	63
	2.00	1.30	0.12	65
	4.00	2.72	0.19	68
	6.00	4.26	0.31	71
60	1.00	0.88	0.14	88
	2.00	1.83	0.08	91.5
	4.00	3.51	0.13	88
	6.00	5.40	0.27	90
90	1.00	1.09	0.06	109
	2.00	2.00	0.12	100
	4.00	3.93	0.12	98
	6.00	5.86	0.13	98

Data are plotted as Fig. 2.

Methods as described in the text.

*Each figure is the arithmetic means of five experiments (triplicate samples for each experiment).

TABLE III. DIFFUSION AND RECOVERY OF AMMONIA FROM BLOOD

AMMONIA ADDED	AMMONIA RECOVERED						ARITHMETIC MEAN	STANDARD DEVIATION	% RECOVERY
	1	2	3	4	5	6			
1.0	0.6	0.65	0.65	0.7	0.65	0.80	0.67	0.07	67
2.0	1.05	1.25	1.2	1.0	1.0	1.25	1.12	0.12	60
4.0	2.15	2.45	2.35	2.05	2.45	2.6	2.38	0.18	59.5
6.0	3.15	3.5	3.0	3.0	3.25	4.0	3.32	0.38	55

Columns 1 to 6 are six separate experiments.

Each figure is the mean of triplicate analyses for a sample of blood.

Figures given are micrograms NH_3 per milliliter blood.

Diffusion time 30 minutes; methods as described in text.

Data are graphed in Fig. 3.

TABLE IV. EFFECT OF TIME AND TEMPERATURE OF STORAGE ON AMMONIA CONTENT OF BLOOD

	TIME BEFORE DIFFUSION					
	10 MIN.		30 MIN.		60 MIN.	
SUBJECTS	0° C.	27° C.	0° C.	27° C.	0° C.	27° C.
Persons With Cirrhosis.—						
GR	2.63	2.57	2.57	3.14	2.53	3.68
PR	1.25	1.71	1.71	2.24	1.67	1.90
ED	1.46	1.33	1.22	1.67	1.88	2.26
ES	1.78	1.82	1.67	2.06	1.55	2.06
WB	1.07	1.07	1.76	2.04	2.40	2.58
JM	1.74	1.85	2.17	2.60	2.06	2.60
Ten Normal Individuals.—						
Arithmetic mean	0.50	0.71	0.77	1.01	1.03	1.65
S. D.	0.22	0.20	0.28	0.27	0.28	0.33

Values are micrograms NH_3 per milliliter blood. Each value is the mean of triplicate

Venous blood samples were divided into two portions when drawn; half was kept at room temperature (27° ± 2° C.) and half was kept at 0° C. Storage time was the time from venipuncture to diffusion.

Data are plotted as Fig. 5.

The ammonia content of blood slowly increases after it is drawn and while awaiting diffusion. Table IV shows that this process is markedly influenced by temperature. All samples showed higher ammonia contents after 60 minutes' storage in ice or at room temperature. The ammonia content of samples which were stored at room temperature increased more than samples which were iced. Blood from normal persons or persons with liver disease showed similar increases when stored at room temperature. Some individuals in both groups showed larger and more rapid increases than other individuals. Only slight to moderate increases in ammonia occur during 30 minutes in ice and this time is sufficient to permit the collection and transportation to the laboratory of a number of samples.

TABLE V. AMMONIA CONTENT OF WHOLE BLOOD, PLASMA, RED BLOOD CELLS, AND LYSED RED BLOOD CELLS

NORMAL INDIVIDUALS	HEMATOCRIT	MCG. AMMONIA/ML. SAMPLE				
		PLASMA	RED BLOOD CELLS	LYSED RED BLOOD CELLS	WHOLE BLOOD	
					EXPERIMENTAL	CALCULATED*
GJ	44	0.83	0.79	1.28	0.80	0.81
HC	49	1.11	0.82	0.84	0.77	0.97
JD	45	1.28	1.13	0.76	0.86	1.21
DS	38	0.51	0.56	1.76	0.77	0.53
MD	34	1.17	0.99	2.68	1.05	1.11
CL	44	0.49	1.03	—	0.99	0.73
BP	45	0.77	0.49	—	0.71	0.64
LB	45	0.72	0.72	—	0.71	0.72

Diffusion was begun one hour after the samples were drawn. An aliquot of whole blood was iced immediately. The remainder was centrifuged at 2,500 r.p.m. for 30 minutes at 0° C., and the separated plasma and red cells were iced. An aliquot of these red cells was lysed by addition of an equal volume of 0.1 per cent Saponin. The values given are corrected for this dilution.

*Whole blood NH_3 =

$$\left(\frac{\text{hematocrit}}{100} \times NH_3 \text{ in R.B.C.}\right) + \left(\frac{100 - \text{hematocrit}}{100} \times NH_3 \text{ in plasma}\right)$$

TABLE VI. COMPARISON OF ARTERIAL AND VENOUS BLOOD AMMONIA CONCENTRATION

NORMAL			PERSONS WITH CIRRHOSIS (WITH AND WITHOUT COMA)		
ARTERIAL	VENOUS	A-V DIFFERENCE	ARTERIAL	VENOUS	A-V DIFFERENCE
0.92	0.94	−0.02	2.10	1.16	0.94
1.02	1.07	−0.05	1.33	1.22	0.11
1.11	1.02	0.09	1.82	1.55	0.27
1.02	1.08	−0.06	1.71	1.17	0.54
0.94	0.89	0.05	1.56	0.90	0.66
1.13	1.24	−0.11	2.19	1.43	0.76
1.11	1.04	0.07	1.95	1.79	0.16
1.24	1.39	−0.15	1.48	1.48	0
0.56	0.92	−0.36			
1.11	1.11	0			
Mean 1.02	1.06	−0.05	1.77	1.34	0.43
S. D. 0.19	0.15	0.13	0.31	0.26	0.34
Range 0.92-1.24	0.86-1.39	−0.36-0.09	1.33-2.19	0.90-1.79	0-0.94

Figures given are micrograms ammonia per milliliter blood. All analyses were begun 30 minutes after blood was drawn. Samples were drawn from same antecubital fossa, venous first, followed by arterial. Blood was at room temperature until analyzed. All samples were run in triplicate. Data are plotted as Fig. 6.

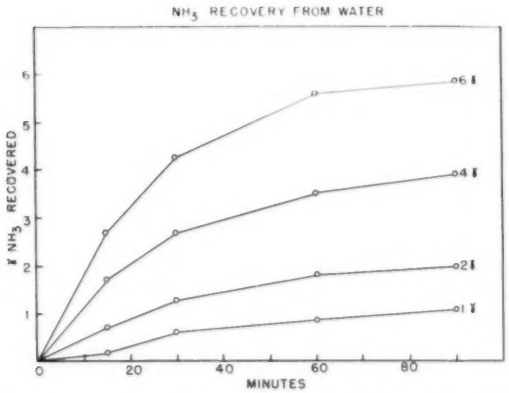

Fig. 3.—Diffusion and recovery of NH_3 from water. Data from Table II. The number at each line is the micrograms of ammonia added.

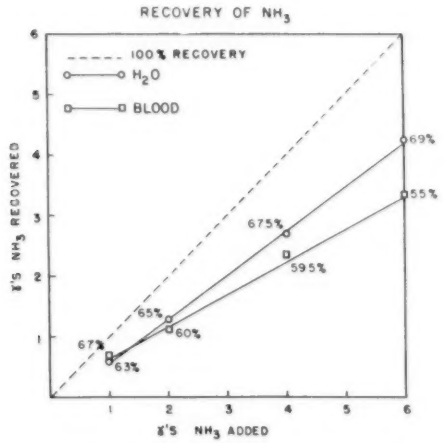

Fig. 4.—Diffusion and recovery of NH_3 from blood and water. Figures shown are per cent recovery. Diffusion time is 30 minutes. Data from Tables II and III.

The storage temperature did not affect the diffusion and recovery of ammonia from water. Standards which were kept in the ice bath or at room temperature for 10, 30, and 60 minutes before diffusion showed identical recoveries.

Hemolysis of blood influences its ammonia content. Data shown in Table V compare the ammonia content of whole blood, plasma, and hemolyzed erythrocytes from the same sample of blood. The ammonia content of plasma and erythrocytes was very similar. Some of the hemolyzed samples showed a considerable increase in blood ammonia. Two per cent hemolysis of some blood samples will increase blood ammonia by 3 to 5 per cent. For greatest precision therefore, hemolysis should be avoided.

Ammonia content was determined in both arterial and venous blood of ten normal individuals and eight patients with hepatic disease (Table VI). The mean value for venous blood ammonia of the patients with cirrhosis was higher

than the mean value for the normal persons. However, four of the eight patients had venous blood ammonia concentrations in normal range. The arterial blood levels of these patients with cirrhosis were all higher than the normal range. Arterial blood levels are more closely correlated with the clinical condition of the patient with cirrhosis than the venous blood concentration. However, the clinical condition of these patients was not always predicted from the arterial ammonia concentration. In Fig. 6 the arterial blood NH_3 is compared with the arteriovenous differences and a significant relationship is apparent. The regression coefficient is 0.64 and it is very unlikely ($p < 0.001$) that the increased uptake at high arterial concentrations is due to chance.

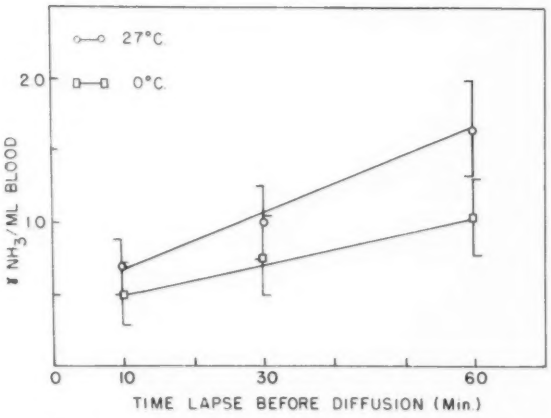

Fig. 5.—Effect of time and temperature of storage on the NH_3 content of blood. Open circles and squares are means of ten normal individuals. Vertical lines show one standard deviation from these means. Data from Table IV.

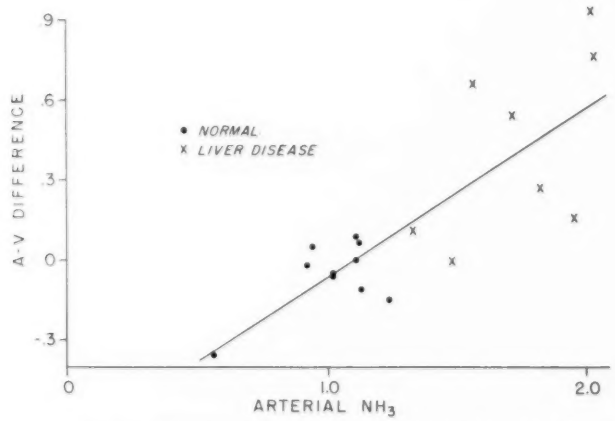

Fig. 6.—Regression of arteriovenous differences on arterial blood ammonia concentration. Figures given are microgram NH_3 per milliliter. Data from Table VI.

DISCUSSION

It is widely known that analysis for blood ammonia is not as satisfactory as most of the methods of biochemistry in common use. Very few of the clinical investigators have stated the precision, sensitivity, and reproducibility of blood ammonia analysis in their laboratory. Therefore, it is difficult to compare these methods.

The colorimetric reaction described has precision, sensitivity, and reproducibility which we were unable to achieve with the widely used Conway method and titrimetric measurement. The weak acids and bases employed in the titrations are easily contaminated by atmospheric carbon dioxide. The end point after titration of a weak base and strong acid is difficult to define with any indicator. Furthermore, colorimetric methods permit rapid and convenient analysis of many samples in triplicate.

Colorimetric analysis of ammonia with Nessler reagent has been widely employed.[4, 5] The sensitivity of the method in various reports varies widely. The colored product is insoluble and forms an unstable colloidal suspension so that color development in different experiments may vary widely. Color development is not always proportional to the ammonia present, particularly at the lower concentrations. The product which is measured has only ¼ to ⅕ the optical density of the present method and the lower limit of accuracy of the Nessler method is therefore much higher than the present method. In addition, some clinical laboratories in which Nessler's reagent is employed note variations of ± 0.4 to 0.5 meg. in duplicate aliquots from the same blood sample. Colorimetric analysis of ammonia with the ninhydrin reaction is satisfactory[2, 6, 7] but not as sensitive as the sodium phenolate reaction. The data in this article show good agreement and high readings with as little as 0.5 meg. of ammonia in 10 ml. final dilution.

Some of the problems in ammonia analysis are common to all methods. Contamination of glassware or reagents with traces of ammonia from the atmosphere, cigarette smoke, etc., is a hazard that requires meticulous cleanliness and constant vigilance.

Problems encountered in blood sampling must be recognized. Patients should be fasting at least 6 hours.[8] Hemolysis may introduce small errors. The arterial blood ammonia is a more reliable indication of NH_3 intoxication than the venous concentration. The arteriovenous differences in ammonia content are most likely a result of detoxication of ammonia in the extremities[5, 9] and the brain.[5, 10, 11] Our data confirm that when blood ammonia concentrations are normal, these arteriovenous differences are not noted, possibly because the "detoxifying" mechanisms have a threshold or the ammonia precursors in hepatic disease are more readily deaminated.

The "normal" concentrations of venous blood ammonia vary widely with different techniques of measurement. The means reported by various investigators ranged from 10 to 120 meg. of ammonia per 100 ml. of blood. Our mean value obtained from venous blood of 20 normal individuals was 94 ± 26.6 meg. per 100 ml., with a range of 46 to 139.

There is considerable doubt that normal blood circulating in vivo contains any ammonia at all[3]; however, all investigators report that blood ammonia appears and increases rapidly after shedding. The precursors of this ammonia and the mechanisms of its release are unknown. Enzymatic deamination of nucleotides and hydrolysis of amides such as glutamine is one source. Doubtless there are other labile ammonia precursors. Conway showed that a CO_2 atmosphere over the sample inhibits some ammonia formation, but this has not been necessary or practicable for clinical purposes and does not completely prevent the formation process. Our data demonstrated that ammonia formation is slowed by icing the sample tubes during collection of a series of samples and transportation to the laboratory. Enzymatic release of ammonia from blood may be prevented by protein precipitation with trichloroacetic acid. The ammonia content of these acid filtrates increases slowly unless they are iced or frozen.[7]

Blood ammonia and tissue ammonia may increase when labile compounds are split by the alkali which is added to make the ammonia diffusible.[3] Under certain circumstances, particularly for tissue analysis, rapid diffusion such as with the Seligson device may be advantageous. This is rapid and convenient and is reported to obtain 95 per cent recovery in 10 minutes. Other investigators have confirmed that diffusion and recovery of ammonia which is added to blood is less complete than ammonia added to water.[3] This has been attributed to differences in rates of diffusion. It could also be due to binding of the added ammonia. This occurs in the presence of alanine and cytochrome C.[12] Bessman and Seligson have also observed apparent binding or "consumption" of added ammonia.[13] There is no enzyme activity in trichloroacetic acid filtrates of blood and the diffusion and recovery from such filtrates and from water is identical.[6]

The concentration of blood ammonia found in any given blood sample depends on the conditions and methods of measurement. It is essential that the methods be standardized and uniform in all experiments and each investigator must establish his own "normal" and "abnormal." The "true" concentrations of ammonia in blood circulating in vivo cannot be stated at present. The contribution of ammonia precursors and the effect of ammonia binding for any given sample are unknown. Fortunately, the simpler distinction of normal and abnormal may be useful in diagnosis and treatment even though these other phenomena are not completely understood. Fairly precise limits can be set for the amounts of ammonia evolved from blood of normal persons provided that the analysis is performed under uniform conditions.

The new colorimetric method has many advantages so that it now appears feasible to measure the activity of certain enzymes by measuring the ammonia which is bound or evolved in the reaction.[12]

SUMMARY AND CONCLUSIONS

1. The colorimetric determination of ammonia with sodium phenolate and hypochlorite catalyzed by nitroprusside is sensitive, reproducible, and con-

venient. Excellent precision is obtained in the range 0.5 to 6 mcg. of ammonia.

2. The factors influencing the amount of ammonia found in blood are discussed. Control and standardization of these factors is essential for the most reliable results. The time between venipuncture and analysis may be extended to 30 minutes without significant increases in ammonia when the samples are iced.

3. Arteriovenous differences in ammonia concentration are greatest with higher arterial NH_3 concentrations. Arterial blood ammonia is more frequently elevated in patients with hepatic disease, but the mental and neurologic status cannot be predicted from the blood ammonia concentration.

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STAPHYLOCOCCUS AUREUS: BACTERIOPHAGE ON PAPER DISKS

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It was postulated that bacteriophage could be impregnated into paper disks which could be used to identify phage types of bacteria in the manner employed for determination of bacterial sensitivity to antibiotics. Disks cut from millipore filter paper were wetted with Staphylococcus aureus 80 bacteriophage at 4° C., lyophilized, stored at room temperature, 4° C., and -70° C., and tested for lytic activity after 1 to 6 weeks. The disks stored at -70° C. were found to retain full activity and sensitivity after 6 weeks. This disk method of determination of phage type of bacteria is believed to have practical application.

BACTERIOPHAGE typing of strains of *Staphylococcus aureus* has become an accepted epidemiologic procedure. However, propagation and standardization of individual phages remains a tedious procedure. Since commercial preparations have become available, phage typing is greatly simplified and, therefore, more applicable to the general bacteriology laboratory.

It occurred to us that phages might be impregnated into paper disks. The disks which carry the various phages could then be used in the manner presently employed for determination of bacterial sensitivity to antibiotics. Mora and Eisenstark[1] have described the preparation and use of disks cut from blotting paper impregnated with bacteriophage. The results of our work in many respects confirm the findings of these investigators, but differ significantly in both methods and materials.

MATERIALS AND METHODS

Phage.—The bacteriophage for impregnating the disks was propagated on a strain of *Staph. aureus* obtained from Dr. Blair[2] and identified by him as phage type 80. The bacteria were grown in heart infusion broth which contained 0.004 mol calcium per liter for a period of 18 hours.[3] The culture was then passed through a Seitz filter and the filtrate was titrated for lytic activity and stored at 4° C.

Disks.—Several types of filter and blotting paper were tested. It was found that the filter paper supplied with millipore filters (A-A-White Grid) was best for carrying the bacteriophage. This paper was also used for the untreated control disks. Disks which measured 6 mm. in diameter were cut from the filter paper and autoclaved.

Incorporation of Phage on Disks.—The sterile paper disks were placed in an Erlenmeyer flask and covered with filtered bacteriophage to a depth of 0.5 inch. The disks and bacteriophage were stored in the refrigerator at 4° C. for 24 hours. The stopper of

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the flask was then replaced by a sterile gauze cover and the bacteriophage was lyophilized* until the disks were dry and well separated. This state was obtained within 30 hours.

The bacteriophage treated disks were divided into 3 lots, placed in tightly sealed ampules, and stored (1) at room temperature, (2) at 4° C., and (3) at -70° C. No drying agent was added. Immediately after drying and again after storage for 1, 2, 3, and 6 weeks at the several temperatures, disks were checked for lytic activity against *Staph. aureus* 80 (the propagating culture) and staphylococcus strains of other phage groups.

Our routine procedure[3] was used for lytic tests. Petri plates which contained heart infusion agar were streaked with young (4 hour) bacterial cultures. The bacteriophage treated and untreated control disks were applied with sterile forceps in the manner used for antibiotic disks. Readings were made after 4 hours' incubation at 37° C. and again after the plates had been left at room temperature overnight.

RESULTS AND DISCUSSION

A clear zone of lysis, approximately 1 to 1.5 mm., appeared under and around the disks which carried phage of the culture type. No reaction developed around the control disks. The diameter of the lytic zone was found to be influenced by the moisture content of the agar. Reduction of the agar content of media from 2.5 to 1.5 per cent increased significantly the size of the lytic zone. Media with agar content of less than 1.5 per cent was too soft and therefore unsatisfactory.

As previously stated, the disks were stored at room temperature, 4°, and -70° C. in tightly sealed ampules. No drying agent was added. There was no difference whatever in the activity of the disks after storage periods of 1, 2, and 3 weeks. In all instances the lytic zones measured approximately 1 to 1.5 cm. in diameter. When the disks were next tested, after 6 weeks storage, it was apparent that the lot stored at room temperature had lost all activity. The disks stored at 4° C. showed slightly reduced activity, exhibiting, however, a surrounding area of plaquing, which measured 1 cm. in diameter and contained over 50 plaques. The disks stored at -70° C. for 6 weeks were as active as when first treated. All results of this procedure of disk typing checked exactly with those obtained by more conventional methods. Five cultures of *Staph. aureus*, including 3 phage types, were spread on agar plates and lyophilized disks 80 were applied. The reactions from use of the disks were identical to those obtained by standard phage typing procedures.

The use of the millipore paper ensures a consistent source for preparation of the disks and absorption of the phage. Titrations could be readily carried out if desired and the diluted phage could be used for impregnation of disks. However, for routine testing the undiluted phage is employed and, therefore, the more simplified method described here would be satisfactory for routine use.

CONCLUSION

Staphylococcus aureus bacteriophage may be impregnated on paper disks cut from millipore filter paper. The paper disks may then be used to identify cultures of unknown staphylococcus in the manner used for determining bac-

*Cryo-evaporator 10-180, Motor Products Corp., N. Chicago, Ill.

terial sensitivity to antibiotics. This simplified procedure gave results identical to those obtained by use of liquid phage. Storage at −70° C. is known to preserve the activity for at least 6 weeks.

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MICROCLOTTING TECHNIQUES

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MANHASSET, L. I., N. Y.

WITH THE TECHNICAL ASSISTANCE OF RHODA ROSENFELD

Therapy with heparin and coumarin anticoagulants is generally controlled by tests using venous blood. To date, micromethods for prothrombin and clotting time estimations have not proved to be as satisfactory. This article analyzes the problems of microtechniques and suggests methods which utilize blood taken by finger puncture and are as sensitive and reproducible as the usual macromethods.

THE adaptation of microtechniques to routine clinical laboratory determinations is becoming increasingly popular. They require relatively small quantities of blood which can often be obtained by finger puncture rather than venipuncture. The basic principles and results of the micromethods are often analogous to those of macromethods which require several milliliters of blood, and the degree of accuracy is comparable.

The adaptation of clotting tests to microtechniques has generally proved to be unsatisfactory. Important variables such as volume to glass surface ratio, tissue fluid contamination, prompt mixing with reagents, and temperature factors have been difficult to stabilize in microtechniques. Even macrotechniques themselves are subject to problems of reproducibility.[1]

The two most commonly performed clotting tests are whole blood clotting time and plasma prothrombin time. This report presents an evaluation of the variables which must be considered in evolving micromethods comparable to the macrotechniques commonly employed for these two determinations.

1. *Clotting Time of Whole Blood.*—Normal blood circulating in normal vessels has a clotting time which is indeterminate. Release of platelet thromboplastic enzymes upon contact with an injured vessel or foreign surface usually initiates the chain of events which leads to clot formation. Silicone coated and similar surfaces are less efficient in initiating these changes than glass. Normal glass test tube clotting times (Lee-White) range from about 5 to 15 minutes, whereas silicone coated test tube times are much longer. Glass capillary tube clotting times are generally about 2 to 5 minutes, whereas the recent use of silicone coated capillary tubes such as described by Glueck[2] yield normal clotting times quite similar to the Lee-White glass test tube times. The data in Table I, columns 1 versus 3, and 2 versus 4, illustrate the differences in capillary clotting time by silicone coated versus nonsilicone coated tubes.

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TABLE I. CAPILLARY CLOTTING TIME COMPARISON OF SILICONE VERSUS UNCOATED GLASS AND FIRST VERSUS SECOND DROP OF BLOOD FROM FINGER PUNCTURE

PATIENT	FIRST DROP SILICONIZED	FIRST DROP UNCOATED	SECOND DROP UNCOATED	SECOND DROP SILICONIZED
1	11 min., 54 sec.	4 min., 30 sec.	4 min., 5 sec.	6 min., 51 sec.
2	12 min., 42 sec.	5 min., 25 sec.	5 min., 2 sec.	8 min., 47 sec.
3	10 min., 15 sec.	6 min., 15 sec.	5 min., 45 sec.	6 min., 11 sec.
4	13 min., 37 sec.	5 min., 5 sec.	5 min., 20 sec.	10 min., 47 sec.
5	9 min.	4 min., 8 sec.	4 min., 28 sec.	tube broke
6	16 min., 30 sec.	5 min., 53 sec.	6 min., 30 sec.	12 min., 6 sec.
7	13 min., 22 sec.	4 min., 25 sec.	5 min., 25 sec.	6 min., 47 sec.
8	11 min., 40 sec.	5 min., 55 sec.	6 min.	7 min., 24 sec.
9	15 min., 2 sec.	4 min.	3 min., 51 sec.	9 min., 47 sec.
Average	12 min., 51 sec.	5 min., 3 sec.	5 min., 7 sec.	8 min., 41 sec.

Room temperature 26° to 28° C.

This table also illustrates another variable, i.e., the nature of the specimen. The first drop of blood from a finger puncture differs significantly from subsequent drops. Contrary to common belief, but consistent with the findings of Glueck,[2] the first drop has a longer clotting time than subsequent drops, and significant variables are less likely to be masked in this first drop. The fact that this difference between first and second drops is clearly detectable with silicone coated capillary tubes (compare columns 1 and 4, Table I), but not with plain glass capillary tubes (columns 2 and 3), demonstrates the importance of confining capillary clotting time determinations to the first drop of blood entered into a silicone coated capillary tube.

The influence of temperature on clotting time is well known.[3] The method of Glueck recognizes the importance of temperature. In her capillary techniques this variable is partially controlled by holding the tube in the palm of the closed hand. However, data presented here confirm that the surface temperature of the hand, even of the same individual, may vary significantly. A series of silicone-capillary clotting times were determined by one technician with the capillary tube and a thermometer held in the hand. Specimens were obtained from normal subjects in different rooms. Hand temperatures ranged from 26° to 34° C. The clotting time results summarized in Table II illustrate the need for strict temperature control. A comparison of capillary tubes rinsed with saline and dry tubes showed no significant difference in clotting time.

TABLE II. RELATIONSHIP OF PALM TEMPERATURE TO SILICONE CAPILLARY CLOTTING TIME IN 79 DETERMINATIONS

TEMPERATURE °C.	NUMBER OF DETERMINATIONS	AVERAGE TIME MIN.:SEC.	AVERAGE DEVIATION
26.0 - 31.9	21	13:00	±1:26
32.0 - 33.9	24	9:16	±1:08
34.0 - 34.9	34	8:02	±1:00

Because of these findings, the Glueck technique has been modified to include more consistent temperature control by the use of a compact portable instrument* consisting of a constant temperature dry metal platform and a stopwatch.

*Prothrometer, manufactured by Oxford Laboratories, Redwood City, Calif.

The final method employed is as follows: (1) Half fill a silicone coated 0.5 by 150 mm. capillary tube* with the first drop of blood from a finger puncture. (2) Place tube on temperature controlled block 5 minutes. (3) Break capillary tube at one minute intervals (2 minutes if subject has been given heparin) until a fibrin strand is apparent. Then break again at about 15 second intervals until two successive breaks show fibrin strands. The time of second break is the clotting time.

When this method was applied to 65 outpatients not on anticoagulant therapy, an average clotting time of 10.06 minutes was obtained, with an average deviation of 90 seconds. Clotting time was repeated on subsequent visits in 21 of these subjects. The correlation coefficient of the paired values of these 21 subjects was significant at the 5 per cent level but not at 1 per cent. These data suggest that the relatively wide range of "normal" clotting times of different subjects reflects in part real differences in the coagulability of the blood, i.e., the wide normal range is not wholly due to uncontrolled variables in the test.

Unlike earlier finger puncture tests, the silicone coated capillary clotting time as described has proved to be adequately sensitive and reproducible to follow heparin therapy. Fig. 1 illustrates the clotting time responses by this method in 7 subjects after a single deep subcutaneous dose of heparin.

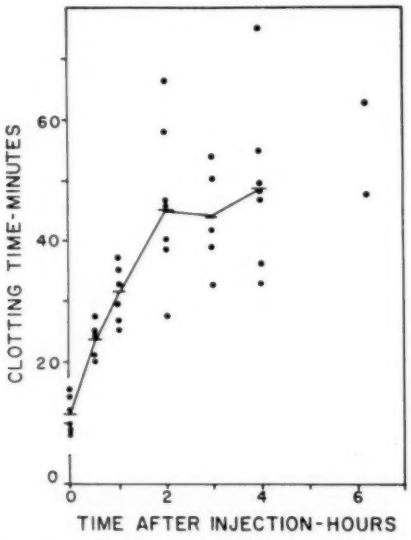

Fig. 1.—Capillary clotting time response of 7 patients to a single injection of 150 mg. heparin into subcutaneous fat.

2. *Plasma Prothrombin Time.*—In this test the amount of surface contact and trace tissue juice contamination are relatively unimportant, since the thromboplastin reagent added in performing the test will largely mask the influence of these variables.

*One hundred fifty millimeter capillary tubes of relatively constant diameter were obtained from Harshaw Chemical Company, Scientific Division, Hastings-on-Hudson, New York, and siliconized by rinsing with aqueous silicone (Clay-Adams Silicad) diluted 1: 100, followed by water rinse and drying.

Most micromethods proposed to date use whole blood instead of plasma.[4, 5] Although these methods have some value, they are not quite comparable to the standard macroplasma techniques. Variations in hematocrit and in citrate-calcium concentration may introduce misleading deviations with the micromethod.

A major problem in developing a microplasma prothrombin technique is the mixing with quantitatively controlled amounts of anticoagulant and the separation of plasma from cells. This was solved by filling capillary tubes to a given point with an appropriate calcium binding anticoagulant, filling the remaining portion of the tube with blood from a finger puncture, capping the tube, and standing it up so that the red cells sediment through the anticoagulant, thus mixing the contents and preventing coagulation. By adding an appropriate amount of dextran to the anticoagulant, sedimentation is more rapid and the need for centrifuging to separate plasma from red cells is obviated (Fig. 2).

The following method was developed and is calculated so that the final reaction mixture contains the same concentrations of plasma and reagents as the usual Quick one stage method.[6]

Standard 75 mm. silicone coated capillary tubes* are marked at 25 mm. at one end and filled to mark with a solution which contains 0.85 per cent sodium citrate and 3 per cent dextran.† The tube is then filled with blood from a finger puncture, via the same end of the tube. The opposite end is capped,‡ and the tube is placed on the capped end for 5 or more minutes. The tube is then broken at a point close to the cell-plasma border. The plasma is delivered into an appropriate vessel at 37° C. with the aid of a length of polyethylene tubing§ or a "vaccination" rubber bulb.[4] The same section of capillary tube is then filled with an equal volume of calcium thromboplastin‖ prewarmed to 37° C., and is added to the plasma in the 37° C. apparatus. Clot formation is timed. We prefer observing clot formation in a small test tube¶ rather than a slide, since the slide may introduce significant surface evaporation, and the end point is more difficult to detect.

Unlike the silicone capillary clotting time previously described, there is no significant difference in prothrombin time between the first versus subsequent drops so that duplicate or even quadruplicate estimations can be obtained from a single finger puncture. In tests performed with citrate without dextran, blood taken after the first drop may sometimes clot in the capillary tube before cell sedimentation accomplished adequate mixing. Such specimens had to be discarded. With the dextran-citrate mixture described this is not a problem.

Table III presents unselected data obtained during one week consisting of 8 studies of normal control subjects and 22 from patients who were receiving anticoagulant drugs. Simultaneously drawn venous specimens were examined

*Blue Tip capillary tubes, Scientific Products, Inc. have a diameter of 1.2 to 1.4 mm.

†See details in legend to Fig. 2.

‡Critocaps, Scientific Products, Inc.

§A size 8 French infant feeding tube was found to fit conveniently over an end of the glass capillary tube, permitting quantitative delivery by gentle blowing.

‖A dry form of calcium thromboplastin reagent such as Simplastin (Chilcott) or Thromboplastinase (Geigy) is used, but is reconstituted in only three-fourths of the volume recommended for standard macroprothrombin techniques.

¶The test tube is 6 by 25 mm., roundbottom, and can be kept in a 37° C. water bath or in a simple adaptation of the instrument previously described.

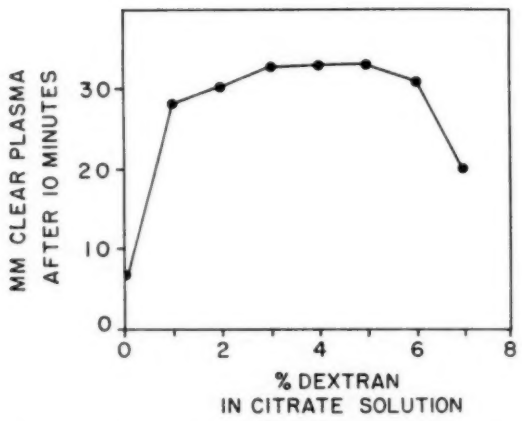

Fig. 2.—Influence of dextran* concentration on speed of sedimation in a 75 mm. capillary tube containing one-third 0.85 per cent citrate and two-thirds blood.
*Henley and Co., Grade H.H. clinical molecular weight 200,000 to 275,000.

by the standard macrotechnique in duplicate in 20 of these 30 studies and were compared with the duplicate or quadruplicate finger puncture capillary plasma prothrombin time estimations. The data (Table III) clearly reflect the essentially identical nature of the macro- and microplasma prothrombin

TABLE III. COMPARISON OF MACRO- AND MICROPLASMA PROTHROMBIN TIME

SUBJECT	MICROMETHOD				MACROMETHOD	
R. R.*	12.5				14.5	
R. R.*	12.5	13.0				
R. R.*	13.5	13.0	13.0	13.0		
R. R.,*	13.5	13.0	13.5	13.5		
R. R.,*	14.0	13.0	13.0	13.0		
N. S.*	14.0	13.0	13.0	13.0		
C. G.*	13.5	14.0	14.0	14.0		
G. M.	14.5	13.5	13.5	13.5	13.0	13.0
N. S.	14.0	14.0				
H.*	15.0	14.0	13.0	14.0		
M. J.	14.5	16.0				
S. J.	16.0	15.0	15.0	15.5	15.0	15.0
La. P.	17.0	16.0	16.5	17.5	16.5	16.0
E. S.	16.0	16.5	17.0	17.5	16.0	16.0
La. P.	17.0	17.0	17.0	17.5	16.0	16.5
D. E. M.	19.5	20.0	20.5	19.5	19.5	19.5
G. M.	20.0	20.0			19.0	19.0
L. A. P.	20.0	20.5	20.0	20.5	18.5	18.5
S. S.	20.5	20.5	21.0	20.0	20.0	20.5
S. S.	22.5	23.0	23.0	23.5	22.0	22.0
L. A. P.	24.5	25.0			24.0	24.0
S. J.	28.5	28.5	28.0	28.5	29.0	28.5
S. S.	28.5	29.0	28.5	28.0	26.5	26.5
G. M.	28.0	28.5	29.0	29.0	28.5	29.0
S. J.	28.0	28.5	30.0	29.0	29.5	30.0
D. E. M.	30.5	30.0			29.5	29.5
D. E. M.	31.0	30.5			31.5	31.0
S. J.	33.0	33.0			33.5	33.5
L. A. P.	34.5	34.5			32.0	32.0
S. J.	36.0	36.5			37.5	38.0

*Normal subjects.

estimations, and the excellent reproducibility of the micromethod. Over 100 quadruplicate determinations have now confirmed these observations.

SUMMARY

These studies demonstrate that reproducible, sensitive, clinically useful estimations of whole blood clotting time by the Glueck method, and plasma prothrombin time by a newly described method, can be performed with finger puncture blood. Temperature must be strictly controlled, and can be accomplished in the home, office, or hospital bedside, or laboratory by the use of a small, dry, portable instrument. Clotting time requires the use of only the first drop of blood from a finger puncture, and a standard silicone coated capillary tube. Prothrombin time requires the use of plasma rather than whole blood, which can be obtained with an appropriate citrate-dextran solution without a centrifuge. Reagent concentrations must be properly adjusted as described for results equivalent to macrotechniques.

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PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

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ABSTRACTS

1. EFFECT OF GUANETHIDINE ON FOREARM VASCULAR RESPONSES TO GRADED DOSES OF NOREPINEPHRINE

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Guanethidine, in some way, interferes with the catecholamine stimulus at sympathetic nerve terminals. Animal experiments suggest that the drug has a reserpine-like action in that it augments the pressor response to norepinephrine (J. A. M. A. 170: 1265, 1959). The present experiments were designed to see if the vasoconstrictor responses as well as the pressor responses to norepinephrine could be increased by administration of guanethidine.

Five healthy young men were studied. Blood flow was measured in the left forearm with a water plethysmograph and blood pressure was obtained from the right arm with a cuff. After control observations, norepinephrine base was infused at rates of 0.075, 0.15, and 0.30 mcg. per kilogram per minute. Each infusion lasted 6 minutes with intervening rest periods of 20 minutes. Flow rates were recorded at about 10 second intervals during the infusions and blood pressure was measured at 30 second intervals. This procedure was repeated in each subject on 3 occasions. The first session (A) served as a pretreatment control. The second session (B) was held 6 days after A; in this interval each subject received 50 mg. of guanethidine twice daily. The last session (C) was held 2 weeks after guanethidine was discontinued.

Control blood pressures were lower and control flows were higher in session B than they were in session A. Norepinephrine caused an increase in blood pressure and a decrease in flow during both sessions. The blood pressure levels attained with each dose of norepinephrine were about the same in both sessions, whereas blood flows as well as decrements in flow were greater with each dose after guanethidine. A consideration of the principle of Laplace makes it apparent that greater active tension must have developed in the walls of blood vessels of larger initial caliber (session B) in response to the same dose of norepinephrine to cause similar or greater reductions in flow. The responses to norepinephrine in session C were similar to those seen in session A.

In these experiments guanethidine increased forearm vascular responsiveness to norepinephrine.

2. CARDIAC CATHETERIZATION FINDINGS INCLUDING SIMULTANEOUS ARTERIOVENOUS SEROTONIN CONCENTRATION IN MALIGNANT CARCINOID SYNDROME

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(INTRODUCED BY PAUL S. HAGEN, M.D.)

The patient was a 64-year-old man with carcinoid syndrome characterized by an intense violaceous flush, diarrhea, and manifestations of severe right heart failure including marked systolic venous pulsations of the neck and liver. A twentyfold increase in 5-OHIAA urine excretion and elevated plasma and serum serotonin levels were repeatedly demonstrated. Cardiac catheterization revealed severe pulmonary hypertension and tricuspid regurgitation. No difference in serum or plasma serotonin concentration was found in samples obtained simultaneously from pulmonary and brachial arteries. Occlusion of the right pulmonary artery did not result in a rise in pulmonary arterial pressure or in pulmonary vascular resistance, suggesting that the pulmonary vascular bed was still distensible. Samples from the brachial artery and from the pulmonary arterial bed distal to the site of the occlusion showed no difference in the serotonin concentration from that of the unoccluded pulmonary artery. No difference in arteriovenous serotonin concentration was found when blood was collected and processed under aerobic and anaerobic conditions. Hyperventilation (Bennett valve) and hypercapnia (5 per cent carbon dioxide inhalation) caused no significant change in pulmonary arterial pressure or serotonin concentration. After obtaining a preliminary dye curve for timing purposes, 7 ml. of saline containing 1.7 million counts per minute of serotonin-C^{14} was injected into the right pulmonary artery. Brachial arterial blood was then sampled continuously during a predetermined 25 second interval. Even allowing for a sizeable error, only one third of the injected radioactivity could be accounted for, the remainder presumably having been removed by the lung.

Reserpine administration for one week resulted in clinically more severe congestive heart failure with a marked increase in right atrial and ventricular pressures as compared to the values observed during the first catheterization procedure. Serum serotonin levels were decreased, whereas plasma concentration remained unchanged during this time and again no significant arteriovenous difference was noted.

These observations emphasize two points: First, since the left heart is exposed to the same serotonin concentration, these studies offer no explanation for the fact that in carcinoid syndrome, lesions are curiously confined to the right heart. Second, reserpine aggravated the degree of congestive heart failure.

3. SOME FURTHER STUDIES ON THE CARDIOPULMONARY EFFECTS OF EXTREME OBESITY

JAMES K. ALEXANDER, M.D., KAMEL H. AMAD, M.D. (BY INVITATION), AND V. WILLIAM COLE, M.D. (BY INVITATION), HOUSTON, TEXAS

The Pickwickian syndrome of extreme obesity has been described as characterized by (1) marked obesity in association with hypoventilation, (2) somnolence, (3) twitching, (4) cyanosis, (5) periodic respiration, (6) secondary polycythemia, (7) right ventricular hypertrophy, and (8) right ventricular failure.

In this study, based on a variety of clinical and physiologic observations in fifty extremely obese ambulatory subjects, the incidence of hypoventilation as indicated by carbon dioxide retention was only 10 per cent. Somnolence was present in one half of the group and was unrelated to the amount of weight gain, the arterial blood oxygen saturation, the central venous pressure, and the level of cerebral blood flow. Carbon dioxide retention was not observed in subjects free of somnolence. However, the degree of somnolence was unrelated to the amount of carbon dioxide retention, and pronounced degrees of somnolence were present without carbon dioxide retention. The arterial blood hematocrit did not exceed 50 volumes per cent in any case and in only 3 instances was the value above 45 volumes per cent. Although pulmonary hypertension was common in this group, it was always accompanied by elevation of pulmonary venous (wedge) pressure in instances where the latter data were available. Frank congestive heart failure occurred in 8 per cent of patients, and, when present, it was associated with symptoms of left as well as right heart failure. Necropsy studies have invariably indicated the presence of combined left and right ventricular hypertrophy with extreme obesity and pulmonary congestion or edema in patients with congestive heart failure.

It has been concluded that the mechanism of the development of somnolence in the obese state is not yet established, that the incidence of carbon dioxide retention is relatively low, and that heart failure, when it occurs with extreme obesity, involves insufficiency of both ventricles. We have not observed a single instance of isolated cor pulmonale in a clinical experience with over one hundred extremely obese subjects over a 4 year period. The Pickwickian combination of hypoventilation, cor pulmonale, and secondary polycythemia must be an exceedingly uncommon accompaniment of extreme obesity.

4. IMMUNOLOGIC STUDIES IN ATHEROSCLEROSIS. III. EFFECT OF ACTIVE IMMUNIZATION WITH POLYVALENT HUMAN BETA LIPOPROTEIN ANTIGENS ON SERUM LIPIDS, PROTEINS, AND ATHEROSCLEROSIS IN CHOLESTEROL-FED RABBITS

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Production in rabbits of antisera to beta lipoproteins from dogs on thiouracil-cholesterol atherogenic diets (Texas Rep. Biol. & Med. 9: 391, 1951; Proc. Soc. Exper. Biol. & Med. 82: 119, 1953) has led to active immunization of rabbits with beta lipoproteins from cholesterol-fed cocks (Lancet 2: 6, 1959). This immunization has been reported to inhibit the development of atherosclerosis subsequently induced by cholesterol feeding in these rabbits.

New Zealand white male rabbits were divided into two groups of 6 each. Six of these rabbits then received weekly intramuscular injections for 4 weeks of polyvalent human beta lipoprotein antigens ultracentrifugally derived from the sera of patients with myocardial infarction. The 6 control rabbits received no injections. During this period, all rabbits were maintained on a stock diet of Purina rabbit chow.

Blood was drawn initially from all rabbits and again after 4 weeks of immunization. All rabbits were placed on a 2 per cent cholesterol diet for 8 weeks and then killed; blood was obtained just prior to death.

Serum gamma globulin and beta lipoproteins increased in 4 of the 6 immunized rabbits after 4 weeks of immunization before the 2 per cent cholesteral diet was instituted. Serum total lipids, lipid phosphorus, total cholesterol, per cent beta lipoproteins, and gamma globulin increased markedly in all rabbits after 8 weeks on the 2 per cent cholesterol diet. Total proteins decreased in two immunized and one control rabbit, largely because of a decrease in albumin (to 2.6 Gm. per cent in one rabbit).

No inhibition of atherosclerosis was observed in this experiment, nor was the severity of gross atherosclerosis quantitatively related to the level of serum total cholesterol or total lipids. The two rabbits that demonstrated the most severe aortic involvement were found in the immunized and control groups, respectively.

It is suggested that active immunization with polyvalent human beta lipoprotein antigens may increase the beta:alpha lipoprotein ratio, while not appreciably affecting total serum cholesterol or total serum lipids. This effect is accentuated with the advent of hypercholesterolemia induced by a 2 per cent cholesterol diet.

5. FAILURE OF THE $Rh_o(D)$ GENOTYPE TO INFLUENCE THE QUANTITY OF ERYTHROCYTE $Rh_o(D)$ ANTIGEN

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(INTRODUCED BY A. I. BRAUDE, M.D., PH.D.)

Hemagglutination studies have suggested that the amount of several surface antigens on erythrocytes is, in part, an expression of gene dosage, and that the homozygote tends to have twice the antigen reactivity as the heterozygote.

In the present study, I^{131}-labeled anti-$Rh_o(D)$ eluates were prepared which specifically bound as much as 70 per cent of the added radioactivity to $Rh_o(D)$-positive cells and only 1 per cent to $Rh_o(D)$-negative cells. The eluates did not bind to other known antigens in commercial erythrocyte typing panels. Under conditions of antibody excess at equilibrium, the relationship between the number of $Rh_o(D)$-positive erythrocytes (and presumably antigen sites) and the uptake of eluate was almost linear.

To compare the amount of $Rh_o(D)$ antigen on D-positive erythrocytes from 159 donors, the amount of eluate bound by 0.5 ml. of a 1 per cent suspension of erythrocytes from each of these donors was related to a standard $Rh_o(D)$ antigen reference curve. The standard $Rh_o(D)$ antigen reference curve was prepared by reacting each eluate with 0.5 ml. aliquots of 0.5, 1.0, and 2 per cent suspensions of $Rh_o(D)$-positive erythrocytes from a standard donor.

One per cent cell suspensions from 145 unselected D-positive donors bound eluate with the effectiveness of between 0.5 and 2 per cent of the $Rh_o(D)$-positive reference cells. The frequency distribution of the eluate binding capacities was monomodal instead of the bimodal distribution which would be expected were there a gene dosage effect. Although cells from fourteen known heterozygotes and eighteen probable homozygotes had a similar fourfold range of eluate binding capacity, there was no indication that cells from either selected group had more or less antigen than did the cells from the unselected donor population.

Statistical analysis of the data indicated the fourfold variation observed in the number of erythrocyte $Rh_o(D)$ antigen sites was valid, but erythrocytes with either high or low eluate binding capacities were not related to the $Rh_o(D)$ zygosity of the donor as proposed by Masouredis (1959).

6. PULMONARY VENOARTERIAL ADMIXTURE IN CIRRHOSIS OF THE LIVER: POSSIBLE SITE OF PULMONARY SHUNTING

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(Introduced by Carleton B. Chapman, M.D.)

Seventeen patients with cirrhosis of the liver and one patient with portal vein obstruction were studied. Oxygen saturation and tension, carbon dioxide tension, and pH of the arterial blood were determined with the patient breathing room air and after 30 minutes of 100 per cent oxygen breathing. The arterial oxygen tension was determined polarographically; the alveolar oxygen tension was calculated from the alveolar equation and the arterial carbon dioxide from the Singer-Hastings nomogram.

On breathing room air, the arterial oxygen saturation ranged between 78 and 94 per cent (mean 89 per cent), and the oxygen tension between 41 and 88 mm. Hg (mean 67 mm. Hg). The arterial carbon dioxide tension ranged between 27 and 47 mm. Hg (mean 35 mm. Hg). The alveolar-arterial oxygen tension gradient ranged between 24 and 75 mm. Hg (mean 44 mm. Hg); this was compared to normal value of 13.0 ± 6.5 mm. Hg.

Breathing pure oxygen resulted in a large alveolar-arterial oxygen tension gradient varying between 60 and 577 mm. Hg with a mean of 324 mm. Hg. This gradient suggested the presence of intrapulmonary (right-to-left) shunting. These shunts ranged between 4 and 29 per cent of the cardiac outputs (mean 18 per cent).

In three patients, blood gas studies made after portocaval anastomosis revealed arterial desaturation and large alveolar-arterial oxygen tension gradients both on room air and on 100 per cent oxygen breathing. These suggested that a decrease in the portal vein pressure did not alter the magnitude of the pulmonary shunts. In the patient with portal vein obstruction and esophageal varices, normal blood gas studies indicated the absence of pulmonary shunting.

The effect of moderate exercise was studied in two patients. An increase in the alveolar-arterial oxygen tension gradient and a decrease in the arterial oxygen saturation resulted, suggesting an increase in the intrapulmonary shunts.

The results suggest that (1) right-to-left intrapulmonary shunting is not directly related to portal hypertension; (2) the site of the shunts, since exercise increased the degree, may well be between pulmonary arterioles and venules and not between periesophageal and pulmonary veins.

7. EXTRAOCULAR MUSCLE, SKELETAL MUSCLE, AND THYROID GLAND MUCOPOLYSACCHARIDE RESPONSE TO THYROID STIMULATING HORMONE

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With the Technical Assistance of Norma Spafford and Elizabeth Linz

Administration of thyroid stimulating hormone to guinea pigs has been reported to result in an increase in metachromatic ground substance in retro-orbital connective tissue and an increase in hexosamine concentration in the retro-orbital contents. Metachromatic crescents have been observed in skeletal muscle of patients with exophthalmic goiter.

One lobe of the thyroid gland, a segment of strap muscle, and the extraocular muscles of one eye of eight dogs were removed surgically, studied histologically, and analyzed chemically. The second lobe of the thyroid gland, extraocular muscles from the second eye, and strap muscle from the other side of the neck were removed after 10 units of thyroid stimulating hormone had been administered subcutaneously daily for 3 to 13 days. Six dogs received saline injections instead of thyroid stimulating hormone. Chemical assay demonstrated an increase in the acid mucopolysaccharide content of each of the three tissues studied in each treated dog, using carbazole and orcinol methods of measurement of the uronic acid component of the polysaccharide. The means of the per cent increase in values were 191 per cent (carbazole method) and 141 per cent (orcinol method) in thyroid gland, 145 and 129 per cent in strap muscle, and 56 and 50 per cent in extraocular muscle. Five totally thyroidectomized dogs that were maintained on thyroxine and that received thyroid stimulating hormone showed similar increases in acid mucopolysaccharide values of 90 and 69 per cent for strap muscle.

Five totally thyroidectomized dogs given no maintenance thyroxine and an additional five totally thyroidectomized dogs given three times the maintenance dosage of thyroxine failed to show an acid mucopolysaccharide accumulating effect of thyroid stimulating hormone.

Determinations done on tissue from six dogs given saline injections in place of thyroid stimulating hormone showed a mean change in acid mucopolysaccharide of +2 per cent in thyroid gland, –14 per cent in strap muscle, and –3 per cent in eye muscle.

These findings with our previous data are evidence that thyroid stimulating hormone activity is associated with widespread alteration in tissues coincident with the accumulation of acid mucopolysaccharide. This effect of thyroid stimulating hormone on muscle appears to be direct and not mediated through the thyroid gland.

8. EFFECT OF VARIATION IN TIME DELAY BETWEEN ATRIAL AND VENTRICULAR CONTRACTION ON CARDIAC OUPUT IN DOGS WITH CHRONIC A-V DISSOCIATION USING ELECTRONIC BYPASS FOR A-V BUNDLE

Franz Bender, M.D. (By Invitation), Arthur H. Kitchin, M.D. (By Invitation), and David E. Donald, Ph.D. (By Invitation), Rochester, Minn.

(Introduced by John T. Shepherd, M.D.)

Dogs with chronic atrioventricular block were anesthetized with morphine and sodium pentobarbital. Catheters were placed percutaneously without thoracotomy to record aortic, femoral arterial, right ventricular, right atrial, and superior vena caval pressures, and indicator-dilution curves for detemination of cardiac output. The ventricular rate was maintained at sinus rhythm by electric stimulation via an electrode at the tip of the right ventricular catheter, each stimulus being triggered by the P wave of the right atrium picked up by a second electrode at the tip of the right atrial catheter. An electronic, variable delay, stimulating circuit interposed between the two electrodes made possible study of the effect of varying the P-R interval on the cardiac output and pressures.

Determinations of cardiac output were obtained in five dogs at 2 and 4 minute intervals after instantaneous changes in the duration of the P-R interval from 0.004 to 0.10 second and in some to longer intervals of 0.20 to 0.80 second. Beat-to-beat analysis of aortic pressure pulses recorded during the period of change indicated a systematic decrease in stroke volume of 5 to 10 per cent when the interval was decreased from 0.1 to 0.004 second and the reverse effect when the interval was increased to 0.1 second. The mean right atrial pressure was slightly and reversibly increased and the end right ventricular diastolic pressure was decreased when the P-R interval was changed from 0.1 to 0.004 second. The changes in cardiac output measured by dye dilution 2 and 4 minutes after these alterations were similar in magnitude and direction to the immediate change indicated by the aortic pressure pulses. Similar studies have been made during decreases in central venous pressure produced by hemorrhage and increases produced by infusion of a 6 per cent solution of dextran.

9. OSMOTIC DIURETIC TREATMENT OF "REFRACTORY" NEPHROTIC SYNDROME

LIONEL M. BERNSTEIN, M.D., PH.D., AND AARON GROSSMAN, M.D. (BY INVITATION), CHICAGO, ILL.

Sixty-five children with the nephrotic syndrome have been observed for 1 to 7 years. Most have responded favorably to therapy with adrenocortical steroids. Fifteen of these patients have been hospitalized 2 to 15 times because of recurring overt nephrotic syndrome, manifested by generalized edema, massive proteinuria, hypoproteinemia, hypoalbuminemia, and hypercholesterolemia. Such cases have been labeled "refractory" nephrotic syndrome.

Mannitol administered intravenously has been used as an osmotic diuretic. Its action depends upon the presence of nonabsorbable particles which retain water and dilute the sodium in the proximal tubular fluid. This reduces the saturation of the tubular cell sites for sodium absorption and results in sodium diuresis. Mercurial diuretics exert a metabolic effect on the renal tubular cells which reduces the avidity of the sodium resorbing sites. Because of their totally different and independent actions, mercurial and osmotic diuretics potentiate each other's effects.

Twenty per cent mannitol was administered intravenously 11 times to nine patients with "refractory" nephrotic edema. The results presented are daily excretions corrected to 1.73 square meters surface area.

In four patients mercurial diuretics alone resulted in urinary excretions of 740 to 2,350 (mean, 1,260) ml. of water; and 30 to 109 (mean, 72) mEq. of chloride; the day after administration of mannitol plus mercurials resulted in excretions of 3,590 to 4,980 (mean, 4,490) ml. of water and 138 to 316 (mean, 219) mEq. of chloride.

In seven studies, water diuresis did not alter sodium or chloride excretions. Water diuresis combined with mercurial diuretics resulted in variable chloride excretion, 7 to 484 (mean, 233) mEq., and water excretion, 1,400 to 5,400 (mean, 3,275) ml. Mannitol combined with mercurial diuretics increased the excretions of chloride to 166 to 531 (mean, 355) mEq., and water to 2,860 to 8,030 (mean 6,015) ml.

Potassium excretions were not significantly altered by water, mercurial, or mannitol diuresis, singly or in combination. Weight (edema) losses during 6 hours of mannitol therapy ranged up to 5 pounds, being determined by balance between excretions and intakes of salt and water.

The combination of mannitol and a mercurial is effective therapy for control of edema in the "refractory" nephrotic syndrome of children.

10. STEROLS IN THE BLOOD OF PATIENTS TREATED WITH MER/29

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(Introduced by Clayton G. Loosli, M.D.)

MER/29 has been shown to cause accumulation of 24-dehydrocholesterol in the blood of two patients with familial hypercholesterolemia (Frantz, Mobberley, and Schroepfer) and one hypercholesterolemic man maintained on a corn oil formula diet (Steinberg, Avigan, and Feigelson). Acceptance of MER/29 as an agent to lower cholesterol requires knowledge as to whether it also produces unusual sterols in the blood of patients without familial hypercholesterolemia on an unrestricted diet. Seven patients hospitalized for chronic disease and maintained on an unselected diet have been given MER/29 (250 and 1,000 mg. per day). Serum extracts have been assayed in duplicate for ferric chloride reacting sterol, sterol producing Liebermann-Burchard color at 420 and 620 mμ, total digitonine precipitable material and total nonsaponifiable material. This combination of methods affords two different means to estimate concentration of noncholesterol sterols and three independent estimates of total sterol content. Sterol other than cholesterol was found in all patients after 2 weeks in amounts which ranged from 8 to 33 per cent of total sterol level. In this period, total sterol showed an average decline of 10 per cent from control levels. Noncholesterol sterol appeared in the circulating blood whether total sterol levels rose or fell and in amounts which appeared independent of MER/29 dose. Similar results were found in two patients with essential hypercholesterolemia and one with biliary cirrhosis. MER/29 appears to be a valuable agent for study of sterol synthesis in living man. Evaluation of its use for long-term therapy requires further knowledge as to biologic effects of sterols other than cholesterol in circulating blood.

11. EFFECT OF DIET ON CREATININE EXCRETION

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(Introduced by Robert E. Hodges, M.D.)

The effect of diet on creatinine excretion was studied in thirty-five male volunteers on metabolic regimens for periods of 2 to 4 months, which included 6 to 12 weeks of formula feeding. Sources of protein in the formulas were casein, skim milk solids, and egg yolks, given singly or in combination. The amounts of protein supplied by the formulas ranged from approximately 20 Gm. per day for 2 men (0.25 Gm. per kilogram of body weight) to 140 Gm. per day for another 2 subjects (2.0 Gm. per kilogram of body weight); 12 men received 115 Gm. of protein daily, and the remainder, 70 Gm. Preformed creatinine was determined in 24 hour urine samples according to the Jaffé reaction.

Depending on protein intake during the control period and the first week on formula, there was no change or as much as a 0.3 Gm. decrease in 24 hour urinary creatinine. During subsequent weeks on formula, creatinine excretion decreased in all subjects, and the extent depended primarily upon the duration of the experiment. Only the very low protein intakes (0.25 Gm. per kilogram)

appeared to augment the diminution in urinary creatinine on formula. These subjects showed drops of 0.8 and 0.9 Gm. per 24 hours by the end of 10 weeks.

It is concluded that the amounts of protein in foods, as used in these studies, appeared to have little or no influence on urinary creatinine excretion, provided the protein intake was adequate. The progressive decrease in creatinine excretion occurred when diets lacked creatinine precursors, regardless of the amounts of protein ingested.

12. CLINICAL VALUE OF ROUTINE SERUM CALCIUM ANALYSIS

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The occurrence of several cases of asymptomatic hyperparathyroidism during studies of hereditary parathyroid adenomas stimulated an interest in evaluating the desirability of performing serum calcium determinations routinely. An ammonium purpurate method adapted for an automatic chemical analyzer was used. During a 14½ month period the serum calcium level was determined on 6,067 consecutive patients presenting themselves for clinical examination. The values ranged from 8.1 through 11.2 mg. per cent in 99 per cent of cases and from 8.7 through 10.5 mg. per cent in 95 per cent of cases with a median of 9.6 mg. per cent. As a result of this study, three cases of hyperparathyroidism and one case of idiopathic hypoparathyroidism were discovered. Also, hypercalcemia of clinical importance was elicited in one case of milk-alkali syndrome, one case of multiple myeloma, and one case of disseminated breast carcinoma. Other patients with suggestively abnormal calcium values are undergoing further investigation. Although the yield of abnormal results appears to be small, a normal result is often helpful in appraising a patient's clinical condition. Therefore, we have concluded that a routine serum calcium analysis, by this method, is valuable in our particular type of medical practice.

13. EFFECT OF INOSINE ON MYOCARDIAL METABOLISM

MARTIN BRANDFONBRENER, M.D., PAUL GORDON, PH.D. (BY INVITATION), AND ROBERT DOWBEN, M.D., CHICAGO, ILL.

Inosine, a purine nucleoside, has been reported to be positively inotropic. Its effects on hemodynamics and myocardial metabolism have been studied in an open-chest, nonfailing dog heart preparation.

Total oxygen consumption, carbon dioxide production, and arterial mixed venous oxygen difference were determined for the measurement of cardiac output. Aortic, and in some cases, left atrial mean pressures were determined.

The left ventricular oxygen consumption, carbon dioxide production, glucose, and nonesterified fatty acid consumption were determined by analyzing arterial and coronary sinus blood and measuring left ventricular coronary blood flow. The latter was accomplished by left main coronary arterial cannulation, using a rotameter, or in some instances by assuming that coronary sinus flow is a constant fraction of the left main coronary arterial blood flow.

Inosine was administered to 21 dogs in 20 mg. per kilogram doses, both intravenously and into the left main coronary artery during a 40 minute period. In 7 animals saline was infused to evaluate the effects of anesthesia and operative manipulation on the preparation.

There was a progressive decline in cardiac output, mean aortic blood pressure, heart work, and left atrial pressure in the control animals. This was not significantly affected by inosine. The myocardial oxygen consumption and carbon dioxide production, as well as coronary blood flow, fell proportionately in both the control animals and the animals that had received inosine; however, the heart RQ increased more in the inosine treated dogs, especially those with initially low arterial glucose concentrations.

The decrease in heart work and myocardial oxygen consumption was accompanied by a fall in nonesterified fatty acid consumption, partially accounted for by a decreased availability of fatty acids. However, glucose consumption was maintained in both the control and inosine treated animals, despite a decrease in heart work.

These data indicate that this inotropic substance does not have a significant effect on coronary resistance or the rate of deterioration of this preparation. A subtle effect on myocardial carbohydrate metabolism is suggested.

14. COLON ANTIBODIES IN ULCERATIVE COLITIS

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Chicago, Ill.

Circulating antibodies apparently directed to colon mucosa were detected in the serum of patients with active ulcerative colitis by double agar diffusion, hemagglutination, antiglobulin consumption, retention chromatography, and immunoelectrophoresis procedures. A slightly acid digest of colon mucosa was employed for the agar diffusion; a saline extract incubated at 37° C. for 24 hours and washed repeatedly was used in the hemagglutination tests. The colon antigens did not react with sera from normal persons or with sera from patients with various diseases, including regional enteritis, systemic lupus erythematosus, and cirrhosis of the liver; positive reactions were obtained in patients with a heterogeneous group of diseases accompanied by abnormal serum proteins. Continued positive hemagglutination tests, after absorption of the colitis sera with different organ extracts and with some intestinal microorganisms, further suggested the colon orientation of the antibodies. The antigenicity of colon was suggested further by the production of colon antibodies in rabbits after injection of guinea pig colon. The circulating antibodies were of low titer, heat labile, and incomplete.

Paper chromatographic studies had demonstrated 15 amino acids in normal and colitis colon; an additional compound, possibly α, ϵ-diaminopimelic acid, a constituent of the cell wall of intestinal microorganisms, in some specimens of colitis colon. Negative aerobic and anaerobic cultures of the colon tissue appeared to eliminate the participation of bacterial cells as such in the antigenicity of colon mucosa. However, the incorporation of α, ϵ-diaminopimelic acid or lysozyme digests of *Bacillus proteus* and spherophorus necrophorus in peptic digests of colon mucosa gave positive hemagglutination reactions with ulcerative colitis serum.

These observations appear to demonstrate the presence of circulating multiple-type antibodies in ulcerative colitis. This immunologic process may reflect a secondary response to the diseased and biochemically altered colon wall; the precise role of this process in ulcerative colitis remains to be investigated. The specificity of the serum reactions in ulcerative colitis remains to be established conclusively.

15. ROLE OF METHEMOGLOBIN IN THE MECHANISM OF DRUG INDUCED HEMOLYSIS ASSOCIATED WITH GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

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(INTRODUCED BY ALF S. ALVING, M.D.)

Current studies demonstrated that primaquine-sensitive Negro males developed higher levels of methemoglobin than primaquine-nonsensitive males after the oral administration of sodium nitrite. This was associated with defective reduction of methemoglobin by primaquine-sensitive erythrocytes in vitro. However, during a primaquine induced hemolysis there was minimal elevation of the methemoglobin level in erythrocytes of primaquine-sensitive males; primaquine-nonsensitive males, who did not undergo hemolysis, developed a fourfold increase in methemoglobin concentration in the erythrocytes. These paradoxical observations were resolved by studies showing greater concentration of methemoglobin in older erythrocytes. The methemoglobin content of older primaquine-sensitive erythrocytes was not demonstrable during administration of primaquine since these older cells were preferentially destroyed by the hemolytic process induced by primaquine.

Further in vitro studies revealed that a metabolic degradation product of primaquine accelerated methemoglobin reduction in primaquine-nonsensitive erythrocytes yet only slightly increased methemoglobin reduction in primaquine-sensitive erythrocytes. The action of the degradation product was similar to methylene blue, an auto-oxidizable dye, which facilitates the reduction of methemoglobin by transferring electrons and hydrogen from reduced triphosphopyridine nucleotide (TPNH) to methemoglobin. The reduction of methemoglobin in primaquine-sensitive erythrocytes by methylene blue or the degradation product of primaquine is probably impaired because of the diminished TPNH. In vitro this degradation product caused a marked fall in glutathione content of primaquine-sensitive but not primaquine-nonsensitive erythrocytes.

It appears then that primaquine or its degradation products induce methemoglobin formation predominantly in the older erythrocytes; simultaneously, the redox degradation product of primaquine can facilitate the reduction of methemoglobin.

16. A STUDY OF ADENOVIRUS INFECTION IN A CIVILIAN POPULATION BY USE OF THE COMPLEMENT FIXATION REACTION

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The study concerns observations made by use of the complement fixation test on the sera of individuals and on serum pools collected in the metropolitan area of St. Louis, Missouri, in the period between 1954 and 1959. Antigens prepared from types 1, 2, 3, 4, and 7 adenoviruses were employed as well as a 6 strain polyvalent antigen. Some degree of type specificity was noted in the single type antigens although cross reactions occurred.

Changes in the level of antibodies in quarterly serum pools collected between 1954 and 1959 from individuals of different ages were observed. Tests with

antigens of types 1 and 2 showed little change between infants and older children. Tests with type 4 and type 7 antigens showed some increase in antibody titer between preschool and school age children. Type 7 antigen was tested against adult sera of all ages up to 77 years. Highest titers were found in young adults. After age 57 years, the age pools were mostly negative for the presence of complement fixing antibodies.

Significant adenovirus antibody increases were noted in approximately 3 per cent of paired sera from adults with respiratory infections tested with the 6 strain antigen, indicating a low percentage of clinical infection. On the other hand, three fourths of the individual sera tested showed the presence of adenovirus antibodies suggesting a high percentage of exposure to these viruses.

Monthly serum pools collected from adults between 1954 and 1959 show that antibody levels as revealed by the reactions to type 1 antigen were higher than with other adenovirus antigens and showed higher titers in the years 1954, 1955, and 1956 than in 1958 and 1959. Tests with type 7 antigen showed increased titers in December, 1957, and February, 1959. Only low titers were found with antigens of types 2, 3, and 4 adenoviruses.

The impression secured from these serologic studies is that a high percentage of exposure to adenoviruses occurs, but that the incidence of clinical infection caused by these viruses is not high in the adult population of the St. Louis area.

17. LOWERING OF SERUM BILE ACID CONCENTRATIONS AND RELIEF OF PRURITUS IN JAUNDICED PATIENTS FED A BILE ACID SEQUESTERING RESIN

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A strongly basic anion exchange resin (MK 135) has been reported by Tennent and associates (Circulation 20: 996, 1959) to form a complex with bile acids which is not absorbable by the intestine.

Oral administration of 10 Gm. of the resin daily to four subjects with jaundice, severe pruritus, and high serum bile acid concentrations (15 to 36 μg per milliliter) was associated with a lowering of serum bile acid concentrations and cessation of pruritus in each patient. Three patients had metastatic carcinoma of the liver and the fourth had biliary cirrhosis.

Serum bile acids were measured spectrophotometrically at their absorption maxima (320 and 380 mμ) in 65 per cent H_2SO_4 (J. Clin. Invest. 37: 1494, 1958). Normal values are: trihydroxy acids 0 to 3.5 μg per milliliter, dihydroxy acids 0 to 1.9 μg per milliliter, total bile acids 0 to 5.5 μg per milliliter.

The patient with biliary cirrhosis had intense pruritus continuously for 6 months. The total serum bile acid concentration was 23.7 μg per milliliter. After taking 10 Gm. of resin daily for 15 days the total serum bile acid concentration fell to 4.1 μg per milliliter and pruritus ceased completely. The resin was then withheld for 11 days; pruritus returned and serum bile acids increased to 20 μg per milliliter. This cycle of alternately giving and withholding the resin was repeated three additional times over a period of 24 weeks paralleled in each instance by greatly increased serum bile acid concentrations with onset of pruritus when the resin was withheld, and a prompt decline in serum bile acids with disappearance of pruritus when the resin was readministered.

There was no significant change in serum bilirubin concentrations or the trihydroxy:dihydroxy bile acid ratio in the four patients during resin administration.

Since the resin combines with bile acids in the lumen of the intestine its ineffectiveness would be expected with relatively complete biliary obstruction

(< 10 mg. fecal urobilinogen daily). This was demonstrated in a fifth patient with deep jaundice, pruritus, and hyperbileacidemia, resulting from an ampullary carcinoma, in whom the resin had no effect on serum bile acid concentrations or the pruritus.

The hypothesis that bile acids cause the pruritus of jaundice is supported but not proved by this study.

18. ALTERATION OF PANCREATIC SECRETION BY ANTICHOLINERGIC DRUGS

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The present status of the effect of anticholinergic drugs on pancreatic secretion remains controversial. Using permanent duodenal fistula dogs, evaluation of the effect of various anticholinergic drugs on secretin stimulated pancreatic secretion has been performed.

It can be demonstrated that equal dosages of single rapid intravenous secretin injections result in reproducible flow rate, volume, maximal bicarbonate concentration, chloride, amylase, and lipase. (Trypsin concentrations are currently under study.) Chloride concentrations vary inversely with the bicarbonate concentrations. This reproducible response was contrasted with response patterns in tandem after secretin stimulation preceded by the administration of various anticholinergic drugs.

The anticholinergic agents studied produced no observable effect on volume and flow rate or bicarbonate or chloride concentration. Interesting alterations in enzyme output were observed. Amylase concentrations decreased precipitously when secretin stimulation was preceded by an anticholinergic agent. Less marked changes occurred in lipase concentrations.

Limited experience with the effect of these anticholinergics on pancreatic response to continuous intravenous secretin administration has revealed results similar to the single rapid intravenous injections.

19. ISOTOPE STUDIES ON THE DEVELOPMENT OF RENAL FAILURE DURING THE TREATMENT OF ASCITES ASSOCIATED WITH CIRRHOSIS

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(INTRODUCED BY W. H. DEARING, M.D.)

Uremia of uncertain cause and indicative of a poor prognosis may occur under various circumstances in patients with liver disease, especially when ascites is present. The development of this complication during the use of a diet restricted in sodium and diuretic agents (mercaptomerin, chlorothiazide, prednisone, and/or spironolactone) for the treatment of ascites was studied in ten patients with cirrhosis by determining changes in total body water, exchangeable sodium, exchangeable potassium, serum electrolyte concentrations, and blood urea values at intervals.

Four patients developed elevated blood urea concentrations (mean increase, +175 per cent) during therapy, but this was not related to the amount or type of drug administered, or to changes in liver function. Initially, total body water,

exchangeable sodium, exchangeable potassium, and blood electrolyte concentrations were comparable with those of the other patients. During treatment, comparable amounts of sodium were lost by uremic and nonuremic patients, but much less water (2.2 per cent in contrast to 8 per cent decrease in total body water to body weight ratio) and less potassium (2.4 per cent in contrast to 3.6 per cent fall in exchangeable potassium) were excreted by patients developing uremia. The degree of hyponatremia developing in uremic patients was twice that in the nonuremic patients, but analysis of the data (Na_S related to exchangeable sodium) showed that this did not reflect sodium depletion and, as changes in serum sodium concentration and the exchangeable sodium to total body water ratio correlated poorly, simple dilution was not the major factor. Hyponatremia was attributed mainly to transfer of sodium from the extracellular to the intracellular compartment, presumably as a result of disordered cell membrane metabolism.

As volumes and sodium contents of the urine were similar in both groups of patients, and as systemic blood pressure did not change appreciably, the retention of water, urea, and potassium appeared to have a renal rather than an extrarenal basis. Changes in glomerular and/or renal tubular cell metabolism, arising in association with more widespread changes in cell membrane metabolism, could therefore have been responsible for the deterioration in renal function.

20. FLUORESCENT STAINABLE ANTIBODIES TO THE EATON AGENT IN HUMAN PRIMARY ATYPICAL PNEUMONIA TRANSMISSION STUDIES

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The association between primary atypical pneumonia and the virus isolated by Eaton in 1944 has been studied recently by Liu using the fluorescent antibody technique. Additional information concerning the relationship of Eaton's virus to human disease has been obtained by retrospective analysis of sera available from two volunteer studies, performed in 1944 by the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board, in which pooled sputa and throat washings from patients with primary atypical pneumonia were administered to forty-eight men, thirty control subjects receiving autoclaved inocula. Lyophilized sera collected before and after inoculation of seventy volunteers were examined for fluorescent stainable antibodies against Eaton's virus.

In the groups receiving active inoculum, 74 per cent had clinical illness and 67 per cent showed fourfold or greater fluorescent stainable antibody rises. Antibody responses occurred in 64 per cent of those with primary atypical pneumonia and 70 per cent of those with a milder respiratory disease. Several control subjects in the first experiment developed illness and/or antibody responses; consequently, the relationship of results to inoculation is not clear. More rigorous isolation and inoculation procedures were instituted in the second experiment. Although no apparent illness developed in the seventeen control subjects, four showed fourfold fluorescent stainable antibody rises. In contrast, eighteen of the twenty-two men who were receiving active inoculum developed fourfold or greater fluorescent stainable antibody responses and fourteen had clinical illness.

Eighty-three per cent of individuals showing fourfold or greater cold agglutinin rises also had responses of fluorescent stainable antibody; 54 per cent of those with fluorescent stainable antibody rises also had cold agglutinin rises.

Cold agglutinin responses were directly related to the degree of illness, occurring in 82 per cent of those with primary atypical pneumonia, in 40 per cent of those with milder respiratory disease, and in 9 per cent who were not ill. Fluorescent stainable antibody responses did not relate to the severity of illness, and occurred in 64 per cent of those who remained well.

With respect to the disease induced in the volunteers, these data suggest (1) an association of fluorescent stainable antibodies to the Eaton virus with primary atypical pneumonia, and with a spectrum of disease ranging down to subclinical infection, and (2) that these antibody rises were of greater specificity than were the cold agglutinin responses. This association does not yet establish the Eaton agent as a cause of primary atypical pneumonia.

21. CERTAIN UNIQUE FEATURES OF LIPOPROTEIN LIPIDS IN CORONARY ARTERIAL DISEASE

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Lipids in serum from subjects in the fasting state and three ultracentrifugally separated serum lipoprotein fractions obtained from forty healthy subjects (20 to 69 years of age) and twenty four patients with coronary arterial disease (39 to 70 years of age) were compared.

The serum lipid concentrations (milligrams per 100 ml.) were significantly higher ($p < 0.03$) in the patients than in the healthy group: cholesterol, 294 versus 247; and phospholipid, 277 versus 246. These differences reflected an increase of these lipids and particularly in the concentration of cholesterol ($p < 0.01$), in lipoproteins of density <1.019 and 1.019 to 1.063; in the former, the cholesterol was 41.7 versus 29.5 and the phospholipid, 37.5 versus 30; in the latter, the cholesterol was 198 versus 153 and the phospholipid, 142 versus 112. In lipoproteins of density >1.063 there were virtually identical amounts of cholesterol, 54.5 versus 54.5, and phospholipid, 123 versus 117. The elevation of cholesterol in lipoproteins of density <1.019 in the patient group appears to be independent of age and the cholesterol content of other lipoproteins.

The cholesterol: phospholipid ratio in whole serum was also greater in the patient group, 1.06 versus 0.999, and was explained by a significant increase ($p < 0.01$) in this ratio only in lipoproteins of density <1.019 (1.14 versus 0.961); this ratio was not different in the two groups in lipoproteins of density 1.019 to 1.063 (1.43 versus 1.44) and of density >1.063 (0.486 versus 0.484).

The whole serum ester: free cholesterol ratios in twelve healthy control subjects and six normo- and seven hypercholesteremic patients were similar (2.17, 2.25, and 2.33, respectively); in the three groups this ratio in lipoproteins of density <1.019 was significantly lower (1.43, 1.55, and 1.46) than it was in lipoproteins of density 1.019 to 1.063 (2.27, 2.10, and 2.65) or density >1.063 (2.94, 3.28, and 2.86).

While it is doubtful that such information is of diagnostic or predictive value, it may have pertinence in the determination of the atherogenicity of these moieties, for, as will be pointed out, there is a provocative similarity in the lipid composition of lipoproteins of density < 1.019 and atheromas.

22. EFFECT OF RATE OF INGESTION OF THE DIET ("MEAL EATING" VERSUS "NIBBLING") ON THE "CONTROL" OF EXPERIMENTAL DIABETES MELLITUS

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(INTRODUCED BY RUTH PICK, M.D.)

Data suggesting that the manner of ingestion of the diet plays a significant role in regulating over-all body metabolism may be summarized as follows: animals fed full spaced meals ("meal eaters") exhibit (1) increased amounts of body fat, (2) decreased body protein, (3) diminished thyroid activity, and (4) altered tissue enzymatic activity, when compared to animals eating the same diet in frequent small feedings ("nibblers"). In view of these findings, it appeared desirable to determine whether eating habits contribute to the regulation of diabetes mellitus; experiments, therefore, were performed to evaluate the relationship of the manner of eating to control of diabetes in rats with alloxan induced diabetes. For this purpose, the food consumption and urinary excretions of glucose and nonprotein nitrogen of rats were measured daily under the conditions of free access to food, commencing approximately one month after diabetes was induced by alloxan. After a week of such observations, the animals were adapted to forced feeding and then fed in this fashion the same quantity of diet daily that they previously had eaten. Insulin dosage was kept constant throughout both feeding regimens. Under the conditions of force feeding ("meal eating"), glycosuria increased by an average of 419 per cent (range, 113 to 762 per cent) and nonprotein nitrogen excretion by 123 per cent (range, 102 to 137 per cent). The same type of data was obtained irrespective of whether the animals were fed a moderate or high carbohydrate diet. Furthermore, when the rats were recycled through the two feeding regimens on two or three occasions, similar results were observed, indicating that the rate of ingestion of the diet, not exacerbations and remissions in the severity of the diabetes, was responsible for our findings.

23. A PROMPT EFFECT OF EXOGENOUS INSULIN ON HEPATIC GLUCOSE OUTPUT IN MAN

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The existence of a direct or prompt effect of insulin on hepatic glucose metabolism is still the subject of controversy. Investigation of this problem in man has been limited by the difficulty of measuring hepatic glucose output directly in human subjects. Measurements of splanchnic glucose output and studies employing isotopically labeled glucose afford only indirect information regarding hepatic metabolism. However, it is possible to estimate hepatic glucose output directly in patients with end-to-side portacaval venous anastomoses, since such an anastomosis separates the hepatic circulation from the blood supply of the remainder of the splanchnic tissues. In such patients blood enters the liver only through the hepatic artery and leaves through the hepatic veins. Peripheral arterial blood, which has the same composition as hepatic arterial blood, can be sampled through an indwelling needle, and hepatic venous blood samples can be obtained by hepatic vein catheterization. The product of the hepatic venous-arterial blood glucose concentration difference and the hepatic blood flow (sulfobromophthalein method) represents a direct measurement of net hepatic glucose output in such subjects.

The effect of exogenous insulin on the directly measured hepatic glucose output was determined in three subjects with hepatic cirrhosis and end-to-side portacaval anastomoses. Six to 10 units of glucagon-free insulin was administered in a single injection into a peripheral vein. During 50 minutes after insulin injection the decreases in the arterial blood glucose concentration ranged from 30 to 60 mg. per 100 ml. Control net hepatic glucose outputs in the three subjects were 128, 41, and 42 mg. per minute. After administration of insulin, the output of the first subject declined to an average of 51 mg. per minute; the other two subjects exhibited average net hepatic glucose *uptakes* of 30 and 6 mg. per minute, respectively.

Under these circumstances a prompt effect of exogenous insulin on hepatic glucose output has been demonstrated in man; in two subjects insulin induced an actual net hepatic retention of glucose. The promptness of the observed decrease in output suggests that insulin may exert its effect directly on the liver.

24. EXCRETION OF CITRATE IN PATIENTS WITH RENAL TUBULAR ACIDOSIS AND NEPHROCALCINOSIS

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(INTRODUCED BY JAMES A. CAMPBELL, M.D.)

Renal tubular acidosis is characterized by a defective ability to excrete a maximally acid urine. Approximately 75 per cent of reported adult cases have had nephrocalcinosis. There are several reports of diminished urinary excretion of citrate in this disease, and it has been suggested that this is responsible for the nephrocalcinosis. Studies of citrate excretion were performed in normal subjects and in patients with renal tubular acidosis to test this hypothesis.

Acute studies of citrate excretion were performed in five normal men. Urine pH varied spontaneously or was altered by ingestion of NH_4Cl, $NaHCO_3$, or $KHCO_3$. The normal urine pH ± S.E. of the mean was 6.48 ± 0.093 and the normal citrate excretion was 0.58 ± 0.090 mg. per minute. Acute studies of citrate excretion in six patients with renal tubular acidosis and nephrocalcinosis were performed after no treatment or treatment with $NaHCO_3$ or $KHCO_3$ for periods up to 2 years. There was markedly diminished citrate excretion in these patients (0.26 ± 0.033 mg. per minute), even though the mean urine pH (7.12 ± 0.011) was higher than that of the normal group. Therapy had little effect on citrate excretion although the systemic acidosis and hypokalemia were corrected. Plasma citrate was normal in all these patients. None of these patients had positive urine cultures at the time of study.

Three patients with other forms of nephrocalcinosis, two patients with chronic glomerulonephritis, and two patients with potassium depletion due to alimentary losses were studied. Those with nephrocalcinosis without tubular acidosis and those with chronic glomerulonephritis had normal or only slightly decreased citrate excretion, whereas the patients with potassium depletion had more significant diminution in urine citrate.

These data support the concept of decreased urine citrate as an etiologic factor in the nephrocalcinosis of renal tubular acidosis, but they do not implicate decreased excretion of citrate as an etiologic factor in other forms of nephrocalcinosis. Because correction of systemic acidosis and hypokalemia failed to improve citrate excretion in renal tubular acidosis and because the plasma citrate was normal, a defect in renal production of citrate is postulated.

25. OBSERVATIONS ON STROKE VOLUME AND VENTRICULAR DYNAMICS IN HYPERTHYROIDISM AND THEIR MODIFICATION BY RESERPINE ADMINISTRATION

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The accelerated cardiac output in hyperthyroidism has been reported to result from an increased heart rate with no essential change in stroke volume. Other available data would suggest that this may not be entirely correct. Recent studies employing Rauwolfia alkaloids in thyrotoxicosis have demonstrated a 20 to 25 per cent slowing of the pulse. It has been assumed that the cardiac output is decreased proportionately.

This study was undertaken to clarify the mechanism whereby patients with hyperthyroidism maintain an elevated cardiac output and to elucidate the pharmacologic effect of reserpine. Eight untreated hyperthyroid patients were studied before and after treatment with 10 mg. intramuscular reserpine daily for 3 days. The supine cardiac output, heart rate, and stroke volume were compared with similar data on eighteen normal male subjects. Six hyperthyroid patients were studied in the passive upright tilt position and the information obtained compared with data on an equal number of normal subjects.

In the supine position the cardiac index (mean 5.82 L.) and heart rate (mean 102) were above normal in all patients. The stroke volume index was also significantly increased (57 ± 10 ml., $p < 0.01$) when compared to normal (45 ± 6 ml.). After administration of reserpine there was a definite slowing of the pulse (85 ± 11, $p < 0.01$) but no change in cardiac index (mean 6.20 L.). Thus, the stroke volume index increased significantly (72 ± 14, $p < 0.02$).

The response to passive upright tilt in patients with hyperthyroidism was similar to the normal. The stroke volume index, however, remained elevated (42 ± 8 ml., $p < 0.05$) compared to the normal (33 ± 6 ml.). After administration of reserpine the disparity was magnified (49 ± 7 ml., $p < 0.01$).

These data demonstrate that augmented cardiac output in hyperthyroidism is affected by increased stroke volume as well as accelerated heart rate. These changes exist in both the supine and upright tilted positions. The acute administration of reserpine has no effect on the cardiac output although pulse slowing is unequivocal. Therefore, stroke volume becomes increasingly important in maintaining the metabolic demands of hyperthyroidism. These data indicate that the minute left ventricular work and left ventricular stroke work index are both increased in hyperthyroidism. Reserpine does not alter the former but significantly increases the work per beat.

26. METABOLIC RESPONSES AFTER STEREOTAXIC LESION IN THE HYPOTHALAMUS OF THE BEAGLE

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The functional relationship between the hypothalamus and the pituitary gland is still not unequivocally determined. With the development of the Gomori staining technique and the identification of stainable granules in the hypothalamus, it has been suggested that the supraoptic and paraventricular nuclei of the hypothalamus are the sites of hormone production for the neuro-

hypophysis and that the posterior lobe is only a storage release center for these hormones. On the other hand, the anterior lobe of the pituitary is united with the hypothalamus by way of the pituitary portal vascular plexus.

Many experiments have been designed to detect the effects of hypothalamic lesions upon various metabolic and endocrine functions. The results of such experiments by various investigators are confusing both as to types of changes which occur as well as the areas in the hypothalamus which are involved.

In 10 female purebred beagle dogs, lesions were placed in different parts of the hypothalamus by the Horsley-Clark stereotaxic instrument. Pre- and postoperative determinations were made concerning the electrolyte, nitrogen, and water metabolism, salt loading, and steroid excretion in all dogs, water loading and renal clearance tests in 2 dogs. Carbohydrate metabolism was studied by glucose tolerance and insulin tolerance determinations in all of the animals. The observations were continued from 2 to 9 months after the lesions were made.

No significant alteration occurred in any of the studies. Two dogs became obese with lesions in the ventromedial area. The water balance of one dog changed from positive to negative after the lesion in the dorsal medial nuclei. Slight changes in the insulin tolerance tests of 3 dogs cannot be considered significant.

Metabolic data and microscopic sections of the hypothalamic tissue are presented.

27. ON THE UPTAKE OF THYROXINE AND TRIIODOTHYRONINE BY RAT DIAPHRAGMS IN VITRO

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CHICAGO, ILL.

Hemidiaphragms from weanling Sprague-Dawley rats were incubated in Krebs-Ringer buffer containing 1.3×10^{-7} M L-thyroxine or L-triiodothyronine labeled with radioiodine. The uptake of triiodothyronine was about twice as rapid as the uptake of thyroxine. When the incubating medium contained 1 per cent bovine plasma albumin in addition to the other constituents, the uptake of both thyroxine and triiodothyronine was depressed but T_3 was again accumulated at about twice the rate for T_4. The data were consistent with a kinetic analysis based on a two step process: (1) simple diffusion into the tissue, the rate limiting step, and (2) binding to cellular proteins, a process which occurs very rapidly compared to diffusion. The diffusion constants were calculated to be: $K_D(T_4) = 0.153$ cm.^{3} per minute and $K_D(T_3) = 0.352$ cm.^{3} per minute. A single species of tissue binding site was found with identical tissue binding capacity and binding affinity for both triiodothyronine and thyroxine. Maximum binding was found to be 28×10^{-4} μM per gram tissue and the affinity constant for binding was 1.4×10^4 Gm. per micromole. The difference in the rate of accumulation of thyroxine and triiodothyronine appears to be due only to the difference in the rate of diffusion. A direct rate equation for the accumulation of thyroid hormone was derived which was integrated. Points calculated by means of this equation fit remarkably well with the experimental data.

28. MECHANISMS BY WHICH CARBOHYDRATE METABOLISM IS ALTERED WITH TRAUMA

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Previous studies have shown that surgical operations, performed on paraplectic persons, below the level of the transected spinal cord will produce alterations in carbohydrate metabolism. This suggested that intact afferent nerve pathways are not necessary to promote a metabolic response to trauma. Other work has demonstrated that injury to a hind leg of a dog will cause an increased secretion of hydrocortisone but that no increase occurs if the leg has been denervated prior to the injury. Since it is theoretically possible that the results found with paraplectic persons could be due to reflex stimulation of the adrenals from the segment of spinal cord distal to the transection, the present study was performed to determine the effect on carbohydrate metabolism of trauma to a denervated leg.

Ten dogs were subjected to amputation of the hind leg except for the femoral blood vessels and nerve and the sciatic nerve. In alternate dogs the nerves were sacrificed. The legs were repaired and the animals allowed to recover 2 or more weeks. One day after a control intravenous glucose tolerance test (24 ml. 50 per cent glucose), the leg was traumatized by an operation distal to the site of "amputation." Glucose tolerance tests were repeated 2 and 24 hours later.

When the effects of nutrition, infection, excitement, blood loss, and anesthesia were carefully controlled, the results demonstrated an alteration in glucose tolerance after trauma, irrespective of the integrity of the nerves. This change was manifest by an elevation of fasting blood sugar, a higher rise, and a delayed rate of fall in blood glucose after the intravenous administration of glucose. In view of these findings, it is questioned whether either afferent nerve stimulation or increased adrenal activity is necessary for the production of abnormal carbohydrate metabolism after injury.

29. RATE OF DISAPPEARANCE FROM PLASMA OF URIC ACID ADMINISTERED INTRAVENOUSLY TO DALMATIAN DOGS

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Dalmatian dogs appear particularly well adapted for the disposition of uric acid. In addition to uricase, a hepatic enzyme for the degradation of uric acid, they also possess unique renal tubules that do not reabsorb uric acid. Therefore, it might be anticipated that Dalmatian dogs would dispose of uric acid more readily than would other dogs. However, this does not appear to be true.

Indeed, we have noted just the opposite, namely that Dalmatian dogs in the fasting state had greater levels of plasma uric acid (0.8 to 1.4 mg. per 100 ml.) than did other dogs (0.2 to 0.5 mg.) and that they removed intravenously administered uric acid from the plasma more slowly.

Uric acid dissolved in lithium carbonate solution was administered intravenously to 4 Dalmatian dogs and to 7 mongrel dogs at the rate of 40 mg. per

kilogram of body weight in 30 seconds. The concentration of uric acid in the plasma, determined by a method employing uricase before and at frequent intervals for 90 minutes after the injection, increased to significantly higher levels in all Dalmatian dogs and remained higher throughout the period of observation. For Dalmatian dogs, the plasma uric acid at 3, 10, and 30 minute intervals after injection measured 19, 13, and 9 mg. per 100 ml., respectively; for other dogs, it was 12, 5, and 2 mg. The plasma uric acid in Dalmatian dogs still was increased notably 90 minutes after injection, whereas it had returned to normal within 60 minutes in other dogs. Concentrations of urea and creatinine in the plasma did not change significantly in either group.

Dalmatian dogs excreted in the urine an increased amount of uric acid after injection; other dogs did not. Hence, differences in renal excretion did not account for the slower rate of urate disposal by Dalmatian dogs. This indirectly suggests that the degradation of uric acid by the uricase mechanism may not proceed as efficiently in Dalmatian dogs as it does in other breeds.

30. PROGESTERONE-ALDOSTERONE INTERRELATIONSHIPS IN PREGNANCY

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Progesterone inhibits the salt retaining effect of aldosterone and desoxycorticosterone in human subjects. It therefore must be intimately involved in the hormonal control of renal sodium excretion during pregnancy. During normal pregnancy the rate of progesterone secretion, as indicated by direct measurements and urinary pregnanediol excretion, increases progressively until near term. A roughly parallel increase in aldosterone excretion occurs in pregnancy.

The addition of 6 to 10 grams of sodium chloride daily to the diets of women in the third trimester of pregnancy lowered the aldosterone excretion from 88 to 59 μg per day in one subject, and from 130 to 106 μg in a second. Urinary pregnanediol declined progressively, reaching lowest levels about the eighth day of salt loading. The control rate of pregnanediol excretion was resumed about 6 days after discontinuing the added salt. In the first subject the urinary pregnanediol decreased from 50 to 36 mg. daily; the second from 42 to 25 mg. per day. The pregnancies were not disturbed by this apparent diminution in progesterone secretion. The added salt was quantitatively excreted in the urine after a lag of 1 to 2 days. Sodium excretion returned to control levels 1 to 2 days after the salt load was discontinued.

Diminished aldosterone excretion during periods of high salt intake has been described in other circumstances, and would be anticipated in pregnancy. The coincident decrease in pregnanediol excretion is surprising, and would cast doubt upon the hypothesis that the rise in aldosterone excretion in pregnancy is simply compensatory for its antagonism by progesterone. The fact that the pregnancy was not disturbed during the high salt intake indicates that the amount of progesterone secreted during pregnancy may actually exceed that which is required to maintain the products of conception and may fluctuate considerably in response to the demands of electrolyte homeostasis. The decreased pregnanediol excretion during salt loading implies that the relative levels of progesterone and aldosterone in pregnancy may in part be determined by a common, yet undiscovered, mechanism. Certainly, an integrating mechanism would be required if homeostasis is to be maintained.

31. RENAL DISTRIBUTION OF SODIUM AND POTASSIUM: RELATIONSHIP TO CONCENTRATING ABILITY OF THE KIDNEY IN POTASSIUM DEPLETED AND VITAMIN D INTOXICATED RATS

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According to present concepts, final urinary concentration is the result of back diffusion of water from the collecting ducts into surrounding renal tissue. Such tissue has been shown to be hypertonic, presumably because the loops of Henle act as a countercurrent multiplier system. Sodium is thought to be transported actively out of the ascending loop and accumulated in medullary tissue. Ullrich and associates found an almost linear correlation between the amount of sodium in the papilla and the osmolality of the urine in normal dogs during various degrees of antidiuresis.

We have estimated the content of sodium and potassium in consecutive segments of rat kidneys, after the administration of Na^{22} and K^{42}, respectively, by comparison of the radioactivity in the segments with the specific activity of the plasma. Urinary osmolality was determined by the Baldes modification of the thermoelectric method of Hill.

In the normal rat the concentration of sodium was found to increase progressively from 65 mEq. per liter tissue water in the cortex to as much as 300 mEq. per liter in the papilla when osmolality of the urine was high. This pattern of distribution was confirmed autoradiographically. In accord with the report of Ullrich's group, there was a direct correlation between the concentration of sodium in the papilla and osmolality of the urine. The concentration of potassium decreased gradually from 115 mEq. per liter in the cortex to 85 mEq. per liter in the medullary tip.

In hydropenic, potassium depleted and vitamin D intoxicated rats with marked impairment of concentrating ability, the relative distribution of both sodium and potassium was not altered significantly. However, associated with the decreased osmolality of the urine was a proportionate or slightly greater reduction in the content of sodium in the papilla. The findings suggest that this failure to establish maximal medullary hypertonicity, as indicated by the diminished accumulation of sodium, plays an important role in the impairment of the urine concentrating mechanism.

32. A METABOLIC DEFECT IN PATIENTS WITH HEPATIC CIRRHOSIS WHICH IS REVERSED BY CYTIDINE AND URIDINE

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Using a new technique for assessing CHO metabolism we have found that sixteen of thirty-eight patients with hepatic cirrhosis have a reversible defect of CHO metabolism, an inability to increase glucose utilization after a small dose of insulin. These findings indicate an impairment of glucose utilization by peripheral tissues and are consistent with studies of Myers showing that patients with cirrhosis have decreased hepatic glucose output.

The technique consists of administering a constant intravenous glucose infusion for one hour (control) followed by an intravenous injection of 3 units of insulin. The specific rate constants for glucose disappearance (K) are calculated for the control period and after insulin administration. In thirty-nine normal subjects insulin caused a mean increase in K of 55 per cent. In the patients with cirrhosis insulin caused a significantly smaller increase (16 per cent). These results document in a quantitative manner the clinical observation that some patients with cirrhosis are resistant to insulin. It has been suggested that the liver secretes a factor essential for the action of insulin on the peripheral tissues of intact animals. We have employed our technique in a search for this hypothetical factor, and have tested certain naturally occurring substances (liver extracts and nucleic acid derivatives) for their ability to reverse the defect of CHO metabolism observed in our cases. Of these, cytidine and uridine, administered together, consistently restored to normal the abnormal response of patients with liver disease. Eight of the latter who had shown a decrease in K values after administration of insulin, exhibited a significant increase in K when 1 Gm. cytidine and 1 Gm. uridine were administered prior to the insulin. Three patients with cirrhosis, who had shown an increase in K with insulin, showed a greater increase in K with cytidine and uridine. None of the other substances we have tested duplicate the action of cytidine and uridine, nor are either one of these nucleosides active by itself. These compounds had no effect on the K values of the two patients with myxedema and the one with idiopathic muscular atrophy who, like the patients with cirrhosis, were unresponsive to insulin.

33. RELATIONSHIP BETWEEN INSULIN TRANSPORTING ANTIBODY AND INSULIN EFFECT

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A poorly understood problem is the diabetic who requires increasingly large amounts of exogenous, foreign insulin. The present study was an attempt to correlate the presence of insulin transporting antibody and the hypoglycemic effect of insulin. A semiquantitative in vivo measurement of insulin transporting antibody was obtained by determining the "insulin space" from levels of radioactivity in plasma taken 30 minutes after intravenous injection of I^{131} insulin. This value was compared with the plasma volume (I^{131} albumin method); the ratio (insulin space : plasma volume) expressed the degree of intravascular retention of labeled insulin. The hypoglycemic response to insulin was determined during a 2 hour intravenous insulin tolerance test. Studies were performed with patients in a fasting state 24 hours after the last dose of insulin.

Of twenty-two patients who had never received insulin (sixteen diabetic and six nondiabetic), the mean insulin space was 4.88 (S.D. 0.89) times the plasma volume, indicating little intravascular binding of insulin. Of twenty-eight diabetic patients who were currently or recently on insulin therapy, the mean ratio was 2.61 (S.D. 1.43) indicating intravascular retention of the injected insulin. In most who were relatively resistant to insulin (requiring 60 to 100 units), the insulin space approached plasma volume (ratio, 1.0). The difference in ratio between the groups was significant ($p < 0.05$). The mean acute drop in blood sugar 20 minutes after injection of insulin was 26.3 (S.D. 11.7) per cent in the first group (no insulin) and 13.8 (S.D. 13.4) per cent in

the second group (on insulin), the difference being significant ($p < 0.005$). An attempt to correlate individual ratios (insulin space : plasma volume) with the entire 2 hour insulin tolerance curve suggested that the higher the ratio the more marked, but transient, the fall in blood sugar; whereas the lower the ratio, the less marked and more prolonged the fall in blood sugar. Three patients who were recovering from acidosis and had again become insulin-sensitive had normal ratios; this suggests saturation of antibody from previous intensive insulin therapy. The technique employed is useful in describing in vivo intravascular binding of insulin, and the findings indicate that intravascular retention of insulin is at least one important factor involved in decreased acute hypoglycemic responses of many patients with diabetes on exogenous insulin.

34. VIRAL PNEUMONITIS IN UNIVERSITY OF WISCONSIN STUDENTS

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An intensive study has been made of university students hospitalized with a nonbacterial syndrome characterized by fever, cough, râles in the chest, and usually by roentgenographic evidence of pneumonitis. This condition has been variously diagnosed as primary atypical pneumonia, viral pneumonitis, virus pneumonia, virus bronchopneumonia, or even acute bronchitis. This clinical picture has been noted in 16.5 per cent of over 700 students admitted to the infirmary with respiratory disease since 1954.

The present study was concerned with the clinical and epidemiologic aspects of 127 patients falling into this clinical category, and on etiologic studies carried out on the paired sera from 115 patients of this group. Primary emphasis has been placed on an evaluation of the primary atypical pneumonia agent isolated by Eaton and associates. Using this method, a fourfold rise in antibody titer to this agent has been demonstrated in 20.9 per cent of the group, and the presence of antibody in titers of 1:10 or higher has been found in 32.2 per cent of the group studied. In 67.8 per cent no antibody was demonstrable. This agent appears, therefore, to be associated with a significant percentage of cases of the virus pneumonitis syndrome, most of which are also associated with the presence of cold agglutinins.

Additional antibody studies have indicated infections with influenza in four patients, hemadsorption viruses in three patients, adenovirus in two patients, and possible infection with histoplasmosis in two patients. Work with the hemadsorption (parainfluenza) group is still in progress. Clinical aspects and epidemiologic infections with these agents will be analyzed and compared.

35. BILE ACID EXCRETION IN MAN AFTER INGESTION OF CORN OIL

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(Introduced by Robert J. Rohn, M.D.)

Diets with a fat content composed largely of unsaturated fats have been efficacious in lowering the serum cholesterol levels of man and animals. At the present time the means whereby this results is not entirely clear. The bile acids have been shown to be important oxidation and excretion products of cholesterol. With the thought that dietary fat might have an influence on bile acid metabolism, the duodenal bile acid content of human experimental subjects was studied after ingestion of diets in which the fat source was varied as to quality.

Bile was obtained by duodenal aspiration after instillation of magnesium sulfate. Bile acids were separated on celite columns into glyco- and tauro-conjugates, and subsequent colorimetric and spectrophotometric determination of cholic, chenodeoxycholic, and deoxycholic acids was made.

In five of six subjects studied, bile acids analyzed after ingestion of a formula diet that contained corn oil as its only fat showed an increase in taurine conjugation relative to control periods. In the total group the ratio of taurine to glycine conjugation rose from 0.49 to 1.13, ratio changes among the individual acids being much less marked. Four subjects ate a general diet during the control period. Two subjects ate a formula that contained heavy cream as its fat source during the control period; both showed increased taurine conjugation.

These results are of interest since they contrast with previous studies made in patients with external biliary drainage and because taurine conjugation of bile acids is predominant in animal species showing a resistance to atherosclerosis.

36. STUDIES ON THE MECHANISM OF LEUCINE HYPOGLYCEMIA

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Administration of L-leucine (0.15 Gm. per kilogram) induces a sharp fall in blood sugar levels in some children with familial hypoglycemia (Cochrane and associates, 1956) and in patients with functioning islet cell tumors (Schwartz and co-workers, 1959), but has no effect in normal subjects. An increase in concentration of plasma insulin in leucine induced hypoglycemia has been demonstrated in familial hypoglycemia and in one patient with an islet cell tumor (Immunoassay, Yalow and Berson, 1960).

In healthy subjects leucine had no effect on blood sugar levels in a dosage of up to 1.37 Gm. per kilogram. On the other hand, intravenous tolbutamide has an acute hypoglycemic effect. After administration of chlorpropamide to nine healthy subjects for several days, marked leucine induced hypoglycemia (L-leucine, 0.2 Gm. per kilogram) could be produced in all of 15 experiments, irrespective of the initial blood glucose level. This was accompanied by increases in plasma insulin-like activity measured by the epididymal fat pad technique. After administration of hypoglycemic amounts of ultra-Lente insulin to nine normal subjects, leucine induced hypoglycemia could be produced in 12 of 20 experiments. Leucine induced hypoglycemia was not as great after insulin as after chlorpropamide but was also associated with increases of insulin-like activity. The hyperglycemic effect of glucagon was not inhibited during leucine induced hypoglycemia.

In two diabetic subjects significant leucine induced hypoglycemia could be induced during chlorpropamide but not during insulin therapy.

In a patient with an insulinoma oral or intravenous administration of L-leucine produced no changes in blood sugar levels or in insulin-like activity. In the same patient intravenous tolbutamide caused profound decreases in blood sugar levels and great rises in insulin-like activity as previously reported.

It is concluded that (1) healthy and diabetic subjects, previously leucine-insensitive, become leucine-sensitive after administration of chlorpropamide, a drug that stimulates islet-cell activity; (2) increased insulin release is the primary mode of action by which leucine causes hypoglycemia in subjects with

augmented islet-cell activity; (3) leucine induces insulin release by a mechanism different from that of tolbutamide; (4) potentiation of insulin activity may be a secondary mechanism of leucine induced hypoglycemia; and (5) the studies reported upon may lead to further advances in our understanding of insulin release and activity.

37. THE RELATIONSHIP BETWEEN RETINOPATHY AND PHYSIOLOGIC TYPE IN DIABETES MELLITUS: PROSPECTIVE STUDY

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(Introduced by Alvin L. Schultz)

A crucial question concerning the serious problem of diabetic retinopathy remains unanswered: Is the retinopathy the result of the metabolic disturbance caused by lack of insulin, or is it an independent event, associated by genetic coincidence with the metabolic disturbance? If the former interpretation is correct, subjects with unstable, insulin dependent diabetes should be predisposed to more severe retinopathy than stable nonketotic patients. Evidence at hand, however, is conflicting and unsatisfactory. Accordingly, a prospective study was undertaken in which patients with diabetes of long duration were selected from a clinic population and were examined for retinopathy. The subjects were divided into groups for sex, age at onset of diabetes, and duration of diabetes. They were classified for physiologic type according to objective criteria derived from standardized tests: subjects showing acute unresponsiveness to tolbutamide with ketosis on insulin withdrawal were classified as "ketotic" diabetics; tolbutamide responsive, nonketotic subjects as "nonketotic" diabetics. One hundred and sixty subjects have been examined.

If subjects of all ages were taken together, ketotic diabetes appeared to be associated with serious, proliferative retinopathy, whereas patients with nonketotic diabetes generally had mild, nonproliferative retinopathy. In young ketotic patients proliferative retinopathy was common and in older, nonketotic patients it was uncommon. However, when patients were matched for age of onset of diabetes, it was only in the group with onset between ages 20 and 40 years that sufficient numbers of both types were available to allow valid comparisons. In this group there appeared to be no difference whatsoever in the incidence of retinopathy between ketotic and nonketotic types.

These results are unexpected and imply that lack of insulin per se does not dispose toward proliferative retinopathy. Instead, it is suggested that increasing age at onset of diabetes in some unknown manner protects the patient against serious retinal disease.

38. SUPERNORMAL PHASE OF ATRIOVENTRICULAR CONDUCTION IN AN INTACT MAMMAL

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Supernormal phase of conduction, first discovered by Adrian and Lucas in 1912, refers to a period in the recovery of exitable tissue during which the response to a stimulus is exaggerated. A number of cases of this phenomenon in man have been described.

The purpose of this report is to present results of experiments in which the supernormal phase of atrioventricular conduction was demonstrated for the first

time in an intact mammal. Mongrel dogs weighing 9 to 15 kilograms were anesthetized with 25 to 30 mg. of sodium pentobarbital per kilogram of body weight and given intravenous potassium in form of isotonic solution of buffered potassium phosphate in distilled water at a rate of 1.0 to 1.2 mEq. per minute. In some the injection of potassium was preceded by administration of acetyl strophanthidin and/or digitoxin. The entire experiment was monitored with an oscilloscope. Supernormal conduction was recorded with remarkable regularity in nearly all of the experiments, regardless whether the potassium was preceded by administration of cardiac glycosides or not.

The supernormal atrioventricular conduction was manifested by (1) the sequence of a short R-P interval followed by a short P-R interval and a long R-P interval by a longer P-R interval and (2) the transmission of an impulse after a R-P interval, the latter if shortened or prolonged would result in failure of conduction of the P wave.

In addition to the shorter P-R interval being coupled to its respective R by a shorter R-P interval, the immediately preceding R-R cycle was prolonged, further supporting supernormal conduction. Normally after a longer cycle the refractory period becomes longer and any change in the subsequent P-R interval is always in direction of delayed and not accelerated atrioventricular conduction.

39. BIOCHEMICAL PROPERTIES OF DIFFERENT ELASTINS

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Two major difficulties involved in biochemical studies of elastic fibers are the constant and intimate association of elastin with other fibrous connective tissue elements such as collagen and reticulin, and the great resistance of elastin to isolation from this connective tissue by any but the most drastic of chemical techniques. This report deals with studies of methods for recovering elastic fibers from different connective tissues by various techniques, and suggests that significant differences in the final product may occur.

One method for isolating elastin is a modification of the classic digestion technique of Lowery and associates, based on alkaline degradation of other connective tissue components. Complete amino acid analyses of base elastins thus obtained from lung and aorta of man and dog are presented.

An alternate procedure, based on proteolytic enzymes which do not attack elastic fibers in the normal state, has been utilized to isolate elastin from lung and aorta. Connective tissue is subjected to alternate digestions with crystalline trypsin and collagenase, until no further protein is liberated. The resultant product, called enzyme elastin, has been isolated from lung and aorta of both man and dog. The amino acid composition of this material is presented.

On comparing the amino acid composition of base and enzyme elastin of dog lung and aorta it is noted that there is a reduction in serine, threonine, methionine, and arginine in the base elastin. In addition there is also a significant loss of aspartic acid, glutamic acid, and histidine. Other studies in this laboratory have demonstrated that there is a striking difference in the rate of attack by the enzyme elastase on base and enzyme elastin. The latter is more resistant to elastolysis by elastase and also by papain.

Amino acid analyses are presented of elastic fibers isolated from normal human lung and from the lungs of patients with tuberculosis, bronchogenic carcinoma, and bullous emphysema. Elastins isolated from normal human aorta are compared with those found in patients with severe atherosclerosis.

These studies suggest that alkaline digestion of connective tissue may result in a product which does not accurately reflect the true composition of elastic tissue.

40. IDENTIFICATION OF ESOPHAGEAL VESTIBULAR DYNAMICS IN APPARENT HIATAL HERNIAS

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(INTRODUCED BY LOUIS RAKITA, M.D.)

The diagnosis of small sliding hiatal hernia is usually made by roentgenogram and is based on the appearance of a pouch which projects above the diaphragm and contains tortuous folds interpreted as gastric mucosa. Are all such pouches truly hiatal hernias or might such as appearance be duplicated by a dilated lower esophageal vestibule? The vestibule is a zone at the distal end of the esophagus manifesting sphincteric characteristics in that a high resting pressure is maintained which relaxes in response to a swallow.

Intraluminal pressures and concomitant roentgenographic examinations were made for seventeen patients with apparent small sliding hiatal hernias. A small triple-lumened, water filled plastic tube was passed via the nose and connected to Sanborn strain gauges. Pressures were measured simultaneously at a distal site and from separate sites, 3 and 6 cm. proximally. A photographic unit recorded the pressures and the exact times of x-ray exposures. The tube was placed so that the middle opening showed a vestibular pressure pattern, there usually being a pattern of peristaltic esophagus above and of stomach below. Barium suspension was swallowed and the pouch previously diagnosed as a hernia was identified.

In four patients the middle tip lay proximal to the pouch and it was felt that a true hernia had been demonstrated. In thirteen patients, however, the middle tip lay within the pouch. In these patients roentgenograms taken at intervals after the barium swallow confirmed the impression that the pouch was the vestibule. Within 1 to 3 seconds after a swallow the pressure at the middle tip dropped—prompt relaxation after a swallow is characteristic of the vestibule. The pouch filled with barium during this period of relaxation and emptied several seconds later when the pressure in the vestibule returned to its high resting level. Tortuous folds which could be interpreted as gastric mucosa were demonstrated in some of these pouches at the time of the study. It would seem, from these studies, that the roentgenographic appearance of sliding hiatal hernia can be mimicked by a part of the esophagus, the vestibule.

41. DEFECTIVE FIBRIN POLYMERIZATION: ITS PATHOGENESIS AND CLINICAL SIGNIFICANCE

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The hemorrhagic diathesis that occurs during states of excessive plasma proteolytic activity ("fibrinolytic states"), particularly such as may develop after surgical operations or obstetric complications (abruptio placenta), has been attributed to a new type of coagulation defect, termed "defective fibrin polymerization" (J. Clin. Invest. 38: 1005, 1959). Preliminary data suggested

that products of fibrinogen proteolysis (formed by enzymatic digestion) interfere with fibrinogen-fibrin conversion through inhibition of fibrin monomer polymerization.

The coagulation anomalies observed clinically or reproduced in vitro by measures enhancing plasma proteolytic activity comprise decreased Factor V, decreased plasma fibrinogen, increased one stage prothrombin time (but normal two stage test), increased thrombin clotting time, and macroscopic differences in clot appearance. In vitro, the last three of these anomalies may be reproduced by adding small quantities of a plasmin or trypsin fibrinogen digest (after enzyme inactivation) to normal plasma.

Ultracentrifugal studies demonstrated that both plasmin and trypsin fibrinogen digests contained a large molecular fragment ($5.25S_{20,w}$) and many small molecular components; the 5.25S component is not digested by either plasmin or trypsin. Ultracentrifugal fractionation of the fibrinogen digest revealed that the 5.25S component was the only one which, upon addition to normal plasma, increased the one stage and thrombin clotting times. The specific inhibitory action of the 5.25S component on polymerization has been demonstrated by a light scattering procedure using fibrin monomer as substrate; the 5.25S fragment does not inhibit thrombin action.

Ultracentrifugal examination of clots (calcium-free) formed in the presence of the 5.25S component (clots later dissolved in urea) showed this component to be incorporated in the clot polymer structure. The data suggest that the 5.25 S fragment competitively inhibits fibrin polymerization through physical interference with normal polymer formation and that the syndrome of defective fibrin polymerization may in part be attributable to loss of clot structural integrity as well as to the coagulation defect.

Defective fibrin polymerization, caused by hyperplasminemia, occurs with relative frequency; its early recognition is desirable since therapy with epsilon aminocaproic acid (an inhibitor of plasminogen activation) has proved useful in its control.

42. DIRECT CARDIAC EFFECTS OF DOPAMINE

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Dopamine (3 hydroxytyramine), a precursor of norepinephrine and epinephrine, has been shown by Horwitz, Fox, and Goldberg to increase the cardiac output of human subjects. The purpose of the present study was to determine the inotropic and chronotropic effects of dopamine in the dog heart lung preparation. These effects were recorded by a strain gauge arch sutured to the right ventricle in 8 of 15 preparations studied.

In each preparation dopamine (100 to 1,500 μg) introduced into the venous reservoir produced a significant increase above the control levels in the cardiac output, the contractile force, the cardiac rate, and the mean arterial pressure. Norepinephrine (50 to 200 μg) subsequently given to the same preparations produced in each animal an increase in each of the four parameters measured.

In 3 of the 15 preparations studied reversal of the sequence of study of these agents also demonstrated significant increase in each animal of cardiac output, contractile force, cardiac rate, and mean arterial pressure with both norepinephrine and dopamine.

By use of a Grass stimulator the cardiac rate was made constant at a level between 222 to 288 per minute. Dopamine (25 to 1,500 μg) injected into the superior vena cava increased above control levels the cardiac output, 53 to 1:1

per cent, the contractile force, 8 to 192 per cent, and the mean arterial pressure, 12 to 31 per cent. Norepinephrine (2.5 to 20.0 μg) subsequently given to this series increased cardiac output 42 to 228 per cent, contractile force 67 to 179 per cent, and the mean arterial pressure 7 to 40 per cent. In each animal a prompt decrease in right and left atrial pressures occurred with both sympathomimetic amines.

It is concluded that dopamine has significant positive inotropic and chronotropic cardiac effects in the dosage range studied. These effects were manifested in the dog heart lung preparation by an increase of ventricular contractile force, a rise in blood pressure and cardiac output, an increase in heart rate, and a fall in atrial pressures.

43. BILATERAL DISTRIBUTION OF INDICATOR IN THE CEREBRAL CIRCULATION OF MONKEYS AFTER INJECTION INTO AN INTERNAL CAROTID ARTERY

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Successful intermittent or continuous determinations of cerebral blood flow by indicator dilution techniques require data concerning completeness of mixing in the cerebral circulation of blood supplying the brain from its multiple sources of arterial inflow. This was studied in seven anesthetized Rhesus monkeys (7.9 to 10.3 kilograms), from four of whom technically satisfactory simultaneous dilution curves were obtained from both jugular bulbs after alternating 0.2 ml. injections of indocyanine green (0.1 mg.) into right and left internal carotid arteries.

Injecting catheters (0.8 mm. outside diameter) were passed under direct vision via the external carotid arteries to the carotid bifurcations. All branches of the common carotid arteries, except the internal, were ligated. Blood was sampled from each jugular bulb at 5 ml. per minute via polythene catheters (1.0 mm. outside diameter) introduced visually via needle punctures into the internal jugular veins. Dead space of sampling systems through the detecting chambers of the densitometers was 0.2 ml. Systemic arterial, central, and jugular venous pressures and respiration were monitored continuously, and cardiac output was determined intermittently by injections of dye in the right atrium with sampling from the femoral artery and one or both jugular bulbs.

Appearance times and contours of dilution curves recorded from ipsilateral and contralateral jugular bulbs after injection into an internal carotid artery were similar. However, areas of contralateral curves usually were less than simultaneous ipsilateral curves, averaging 73, 56, 91, and 143 per cent for 44 right, and 75, 65, 55, and 65 per cent for 41 left carotid injections in four monkeys, respectively. Standard deviations of individual values averaged 13 (range, 6 to 23) per cent of the ipsilateral curve. Mean areas after LIC injections were greater by 13 to 26 per cent than for RIC injections in three animals, and less by 67 per cent of the LIC area in one animal.

Calculated average values for cerebral blood flow by the Stewart-Hamilton equation from the mean areas of simultaneous bilateral curves averaged 145 ml. per minute or 17 (14 to 22) per cent of the cardiac output. These results are not discouraging in regard to developing a method for cerebral blood flow with the use of bilateral jugular sampling after injection of indicator at multiple sites in the arterial inflow to the anthropoid brain.

44. IN VIVO UTILIZATION OF TRITIATED THYMIDINE AS A STUDY OF BONE MARROW ACTIVITY

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A sensitive and rapid method of measuring in vivo changes in tissue and bone marrow nucleated cell metabolic activity as reflected by utilization of tritiated thymidine (H3T) is proposed. This is analogous to the measure of changes in erythropoiesis by radioiron.

Replicate groups of rats were given intravenously 20 μc of H3T (1,100 mc. per millimole) at varying times prior to sacrifice, and plasma clearance studies and tissue H3T uptake in bone marrow, spleen, thymus, liver, and buffy coat were determined. Tissues were homogenized, extracted with alcohol, and counted with diatal-toluene in a liquid beta scintillation counter. Tissue radioactivity was expressed as counts per gram of tissue and marrow as radioactivity per total nucleated cell counts.

Rapid thymidine plasma clearance was noted with a half-life of about 2 minutes. A secondary plasma peak was noted after 30 minutes and proved to be largely conversion to tritiated water. Normal curves of tissue and marrow activity were established and prominent liver pooling was noted.

After the infusion of a cytotoxic agent (cyclophosphamide, 200 mg. per kilogram), a delay in plasma clearance is noted as well as a change in distribution of H3T activity. During the initial 24 to 48 hours there is decreased uptake of H3T. Beyond this time uptake increases markedly with the most prominent rise noted in the marrow sites where better than twofold levels over control values are recorded by 72 hours after infusion of the cytotoxic agent. In the long-term follow-up of animals that were receiving a cytotoxic agent a peak in marrow H3T incorporation was noted at 72 to 96 hours with a rise in buffy coat counts at 168 hours. Peripheral blood values returned to normal at 216 to 240 hours. The level of counts in marrow and buffy coat correlated well with the degree of damage with variation in dosage of the cytotoxic agent.

These observations support the usefulness of H3T in vivo as a measure of nucleated cell activity and reflect a pattern of increased marrow regeneration with damage. Changes in H3T activity precede changes in absolute cell counts.

45. PHASE AND ELECTRON MICROSCOPY OSMOTIC FRAGILITY AND THROMBOPLASTIN GENERATION STUDIES OF PLATELETS IN PATIENTS WITH THROMBOCYTOPENIA

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Parallel experiments with the same fresh materials were performed in six patients with thrombocytopenia and in control subjects in each instance. Those studies included complete coagulation profiles, thromboplastin generation test (TGT), and the crucial techniques of phase and electron microscopy as well as osmotic fragility of the platelets (by means of TGT) at normal and increasingly hypotonic solutions of saline (0.85 per cent to distilled water). Normally platelets appear like "spiders" in 0.60 per cent, "swordlike" at 0.40 per cent "clumped" at 0.34 per cent, and finally, "ghostlike" or as "bubbles" at 0.22 per cent N saline to distilled water. The osmotic fragility of normal platelets in the thromboplastin generation test shows good thromboplastic function after 0.40 per cent N saline.

The significant findings in our studies consisted of early spider formation at 0.85 per cent, clumping and destruction at 0.6 to 0.4 per cent, poor tentacle or swordlike formation at 0.4 to 0.22 per cent, and a surprising persistence of spider intact forms at 0.1 per cent N saline solution and distilled water. This biphasic population of the patients' platelets was paralleled by early thromboplastic activity at 0.85 to 0.6 per cent, then poorer activity at 0.6 to 0.34 per cent N saline, but renewed activity toward distilled water.

These findings were then related to the abnormal thromboplastin generation test found at routine and concentrated levels of patients' platelets and only when their own serum and plasma were utilized. The abnormalities were corrected by the substitution of either normal serum, $BaSO_4$ plasma, or even the serum derived from reclotting the patients' platelet-poor plasma in the presence of their own washed platelets.

The relationships between cytologic and thromboplastic abnormalities were found provocative, in that possible interplay of these abnormal platelets to plasma-serum defects, inhibition and reactivation, could be postulated.

46. SERUM CHOLESTEROL: RELATION OF DEGREE OF ESTERIFICATION TO LIPOPROTEIN DENSITY AND TO LIPOPROTEIN CONCENTRATION AS INFLUENCED BY GONADAL STEROIDS

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The degree of esterification of serum total cholesterol is regarded as an index of hepatic function. Since cholesterol circulates as a component of larger (lipoprotein) molecules, it was of interest to determine the degree of esterification of cholesterol found in the major lipoproteins isolated ultracentrifugally: (1) very low density, $d < 1.006$ Gm. per milliliter, (2) low density beta, $d > 1.006 < 1.019$ Gm. per milliliter, (3) beta, $d > 1.019 < 1.063$ Gm. per milliliter, and (4) alpha, $d > 1.063$ Gm. per milliliter. In thirty-nine healthy men and women, 12 to 96 years of age, the degree of esterification increased with increasing lipoprotein density, irrespective of age or sex. Differences between lipoprotein fractions were highly significant: very low density lipoprotein, 57.8 ± 1.6 per cent; low density beta lipoprotein, 66.1 ± 2.3 per cent; beta lipoprotein, 75.6 ± 0.4 per cent; alpha lipoprotein, 81.3 ± 0.7 per cent; whole serum, 74.5 ± 0.4 per cent (mean values $\pm$ standard errors).

The degree of esterification of serum total cholesterol in health therefore is at least partially dependent on the relative amounts of the major lipoproteins present. The reduction in concentration of serum alpha lipoprotein (cholesterol highly esterified) by testosterone leads to diminished per cent esterification of serum total cholesterol, irrespective of whether serum total cholesterol increases, decreases, or remains unchanged. In a young man (G. M.) methyltestosterone reduced alpha lipoprotein concentration but increased beta relatively more. Serum total cholesterol increased (208 to 321 mg.) and per cent esterification fell (70 to 65 per cent). In an elderly woman (L. R.) methyltestosterone reduced alpha lipoprotein concentration without influencing beta. Serum total cholesterol fell (144 to 103 mg.) and per cent esterification fell (76 to 69 per cent).

Additional studies in more than twenty-five subjects have revealed significant correlation between ester cholesterol concentration and alpha lipoproteins in untreated and androgen or estrogen treated healthy subjects. Increase in alpha lipoprotein concentration by estrogen administration does not cause increased per cent esterification of serum total cholesterol because estrogen decreases per cent esterification of beta and very low density lipoprotein cholesterol.

These observations provide additional evidence of the biochemical individuality of the major serum lipoproteins. They indicate also that the lipoprotein altering effects of gonadal steroids tend to be not only quantitatively opposite but qualitatively dissimilar.

47. ENDOTOXIN SHOCK IN THE PRIMATE

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The hemodynamics of bacteremic shock are poorly understood. Variations between species are such that the dynamics in patients cannot be inferred from those noted in common laboratory animals. Accordingly, endotoxin effects are being studied in the Rhesus monkey.

Femoral arterial pressure, central venous pressure, and cardiac output have been measured in seven monkeys anesthetized with sodium pentobarbital before and after the intravenous injection of 5 mg. per kilogram of endotoxin from *Escherichia coli* (Difco). Cardiac output was determined by the indicator dilution technique. In three animals the chest was opened to record, in addition, pulmonary arterial and left atrial pressures.

Femoral arterial pressure fell within the first 5 minutes (average 28 per cent) and averaged pressures fell slowly thereafter. In three animals the fall at 5 minutes was more pronounced (average 49 per cent) and one of these showed no tendency for the femoral arterial pressure to return to the previous level. Six animals died after 2 to 20 hours, and one was killed after 7½ hours. The average cardiac output fell slowly to 50 per cent at 2 to 3 hours and in all but two experiments had started to fall within 15 minutes. It fell less than femoral arterial pressure, so that calculated total peripheral resistance fell in all animals (average minimum 54 per cent of control). It tended to return toward control levels after several hours. The central venous pressure decreased slightly in four of seven animals during the later phases. Left atrial pressure fell slightly in three of three experiments. Pulmonary arterial pressure rose significantly during the first 15 minutes in two of three trials.

In contrast to most other laboratory species but in accord with clinical observations, the femoral arterial pressure fall was gradual. The decrease in total peripheral resistance has been noted in patients in bacteremic shock and after pyrogens. It occurred despite a fall in cardiac output. The falling cardiac output is probably due to a diminished venous return in view of the tendency for central venous pressure and left atrial pressure to fall, and also since infusions of dextran or blood after endotoxin produced a rise in cardiac output in four experiments. It is possible that the fall in total peripheral resistance may contribute toward pooling of blood, which, in turn, may diminish the venous return.

48. THE PRECIPITINS IN BRUCELLOSIS

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The presence of serum precipitins has been evaluated in experimental and clinical brucellosis as a test for the activity of infection. Six rabbits infected with *Brucella melitensis* were followed up 6 months for serum Brucella precipitins by the Ouchterlony technique. The antigen used was the supernatant of

mechanically disrupted homologous *Br. melitensis* cells. The results indicated the development of at least 10 antigen-antibody systems in the serum of animals with Brucella spleen and liver abscesses. Animals not so affected had a lesser number of serum precipitins, which tended to diminish or disappear in the later months of observation.

The sera of twelve patients with histories of brucellosis were similarly tested for Brucella precipitins. Two patients with spleen and renal *Br. melitensis* abscesses and one patient with acute brucellosis showed at least one or two precipitins in the sera. Nine other patients, three with acute brucellosis and six with histories of brucellosis dating back 5 to 10 years, did not have serum Brucella precipitins.

The Brucella extract used in the precipitin tests was fractionated on a DEAE cellulose column. Seven peaks of activity were detected spectrophotometrically to wavelengths of 260 and 280 Å. Precipitin tests with the Brucella infected rabbits' sera and the separated antigen fractions indicated precipitin formation to the components of four of these peaks.

The results suggest that the precipitin test may be a valuable tool in the diagnosis of active Brucella infection, particularly in chronic cases with abscesses. Although antigenicity of Brucella cell walls has not been unequivocally ruled out, most if not all the experimentally induced Brucella precipitins were formed against antigens found in certain fractions of soluble "bacillary juice." This would suggest that only in severely infected cases where whole bacterial cells can be degraded would precipitins be detectable.

49. RENAL LESIONS INDUCED BY HETEROLOGOUS INSULIN: AN EXAMPLE OF FOREIGN PROTEIN NEPHRITIS

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(INTRODUCED BY HARRY F. DOWLING, M.D.)

The object of this study was to investigate the effect of repeated injections of insulin upon kidney structure. Ten rabbits were injected intravenously with short- and long-acting preparations of commercial insulin in doses of 8 to 16 units per injection. The animals were killed 4 weeks after a total of 66 injections had been given, or 24 weeks after initiation of treatment. For comparison, ten rabbits were given injections of normal human sera and nine served as untreated controls.

Microscopic lesions occurred in the kidneys of 80 per cent of the animals treated with insulin, in 100 per cent of those given human serum, and in none of the nine untreated controls. The earliest changes were stasis within the glomerular capillaries which were packed with red blood cells and a protein coagulum. In one half of the animals treated with insulin, definite pathologic glomerular changes were present consisting of either focal or diffuse hypercellularity. In these glomeruli, the proliferation of epithelial cells often obscured the outlines of capillary loops. Associated findings were exudation of protein into Bowman's space with early crescent formation, and hyaline casts within the tubules; periglomerular fibrosis also was observed. In one animal, a PAS-positive glomerular nodule resembled the characteristic human lesion described by Kimmelstiel and Wilson.

Among the animals treated with insulin and serum focal interstitial changes were found in 60 per cent. Irregularly arranged nuclei from disintegrated tubules were associated with infiltrates of lymphocytes and plasma cells. Also microscopic pitting scars developed among some of the animals treated with

insulin. The location of interstitial foci was topographically unrelated to the significant glomerular lesions.

The experiment suggests that the prolonged administration of heterologous insulin produces renal lesions. The histologic changes resemble those observed after repeated injections of human serum or other foreign protein into rabbits. Preliminary observations also suggest that antibodies to insulin developed in the animals treated with insulin. The data are compatible with the hypothesis that the prolonged use of insulin in diabetic patients produces renal lesions; this may represent a human analogue to the artificial model of foreign protein nephropathy produced experimentally in animals.

50. COMPARISON OF BACTERIOSTATIC AND BACTERICIDAL ACTIVITY OF PENICILLIN, ERYTHROMYCIN, TETRACYCLINE, AND CHLORAMPHENICOL IN HUMAN SERUM

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WITH THE TECHNICAL ASSISTANCE OF REBECCA DRAGOS

(INTRODUCED BY OSCAR M. HELMER, PH.D.)

Antibiotics are bacteriostatic in low concentrations and bactericidal at higher. These end points are influenced by susceptibility of the organism to the antibiotic and variations in the nutrient media. Recognition and control of these factors permits comparison of the relative antibacterial activity of antibiotics.

Penicillin, erythromycin, tetracycline, and chloramphenical, the most commonly prescribed oral antibiotics, were included in this study.

Twenty-nine penicillin-sensitive coagulase-positive *Staphylococcus aureus* strains with different phage patterns were obtained from infected patients. These were subjected to three serial transfers at 24 hour intervals to obtain a rapid rate of multiplication. An 18 hour culture (0.04 ml. of a 10^{-3} dilution) was inoculated into test tubes that contained serial twofold dilutions of the antibiotic. Dilutions of the inocula, antibiotic, and the serial twofold dilution were made in human serum. The sterile pooled human serum for this study was heated to remove complement and adjusted to pH 7.4 with acid at room temperature, under vacuum.

After incubation for 48 hours, the bacteriostatic end point was established as the first clear tube preceding the concentration that showed visible growth. All clear tubes were subcultured on nutrient agar. After 48 hours' incubation, the bactericidal end point was established as the lowest concentration which showed no growth on subculture.

The results of the study indicate that to achieve inhibition and killing of the staphylococci, tetracycline and chloramphenicol must be present in higher concentration than is needed with penicillin and erythromycin.

With the usual average dose of these oral antibiotics, the peak concentrations achieved in the patient's serum after addition of chloramphenicol and tetracycline would be bacteriostatic against some of the staphylococcal strains but bactericidal against none. Erythromycin would be both bacteriostatic and bactericidal against most of the staphylococci. Penicillin is bacteriostatic and bactericidal for almost all strains studied.

We must conclude from our studies that penicillin is still the most effective orally administered antibiotic against sensitive *Staph. aureus* strains, and erythromycin is a close second; tetracycline and chloramphenicol are recognized to be poor antistaphylococcal antibiotics.

51. NOSOCOMIAL URINARY TRACT INFECTIONS: ANALYSIS OF FACTORS INFLUENCING THE DISTRIBUTION AND ANTIMICROBIAL RESISTANCE OF UROPATHOGENS

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(Introduced by W. F. Scherer, M.D.)

A change in the pattern of antimicrobial resistance and an increase in the number of drug-resistant bacterial strains infecting the urinary tract have suggested to some that a "hospital" population of resistant gram-negative organisms was emerging in a fashion similar to that observed with staphylococci. To test this thesis, an unselected sample was obtained of bacterial strains infecting the urinary tracts of patients at The Johns Hopkins Hospital. The infections in 230 patients were studied with regard to (1) locale of acquisition (whether contracted in or out of the hospital), (2) presence of structural abnormality of the urinary tract, (3) prior instrumentation or surgical manipulation of the urinary tract, and (4) antecedent antibiotic administration. Bacteriostatic and bactericidal end points against 10 antibiotics were obtained by the tube dilution technique on 300 pathogens isolated from these patients. It was found that a majority of uropathogens was acquired in the hospital, and the differences in distribution of genera between home and hospital were significant ($p = 0.013$). Pseudomonas species was hospital-acquired and Proteus species nonnosocomial in origin, whereas strains of *Escherichia coli*, Klebsiella, and paracolon were acquired almost equally in and out of the hospital. Acquisition in the hospital appeared strikingly related to instrumentation of the urinary tract. In nearly all instances, urinary pathogens acquired in the hospital were no more resistant to antibiotics than were nonnosocomial strains. Structural abnormality predisposed patients to infection, but no bacterial genus tended to infect a structurally altered urinary tract more frequently than another. Previous chemotherapy did not remarkably alter the genus distribution of these uropathogens, but it increased antimicrobial resistance for Klebsiella (only to tetracycline and streptomycin) and paracolon (to tetracycline and chloramphenicol).

Thus, it appears that the situation involving gram-negative urinary pathogens is not analagous to that of the staphylococcus. The results of the present study indicate that, in general, a hospital population of more drug-resistant gram-negative uropathogens has not become apparent.

52. EVALUATION OF TESTS FOR THE RHEUMATOID FACTOR

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An analysis of experience over an 8 year period with three commonly used tests for the rheumatoid factor involving approximately 8,500 sera is presented. The sheep cell agglutination test (SCA-Ziff) (3,000 tests), the bentonite flocculation test of Bozicevich and associates (3,200), and the slide latex test (Hyland Laboratories) (2,500) have been evaluated as to incidence of positive reaction in rheumatoid arthritis and other rheumatic diseases. In most instances at least two of these tests were performed on the same sera. The sera were obtained from patients presenting with a wide variety of rheumatic symptoms in which the diagnosis was moderately well established by many different physicians working in a university clinic or hospital.

The sheep cell agglutination test was positive at least once in 72 per cent of the patients with rheumatoid arthritis, the bentonite flocculation test in 67 per cent, and the slide latex test in 68 per cent. These values are somewhat lower than in reports of comparable series. This difference is attributed to the lack of uniform criteria used in making the diagnosis. In all three tests the incidence of positive reactions for Still's disease and psoriasis with arthritis was extremely low.

False positive reactions with the sheep cell agglutination test occurred in 16 per cent in rheumatic diseases other than rheumatoid arthritis and 8 per cent in patients with no objective evidence of joint disease; corresponding results with the bentonite flocculation test were 5 and zero per cent and with the slide latex 9 and 3 per cent, respectively.

When the sheep cell agglutination and bentonite flocculation tests were compared in 312 identical sera there was agreement in 90 per cent. In this group of tests when there was disagreement the bentonite flocculation showed a slight increase in sensitivity with a corresponding decrease in specificity. The sheep cell agglutination test was discontinued in 1958 because of the laboriousness of the test. Since that time the bentonite flocculation test has been compared with the slide latex test in 1,185 identical sera. In this comparison there was agreement in 96 per cent without appreciable differences in sensitivity or specificity.

These results indicate no significant differences in reliability among these three tests. The simplicity in performing the bentonite flocculation and slide latex tests offers a distinct advantage for routine use.

53. EFFECT OF L-ARGININE ADMINISTRATION ON RENAL EXCRETION OF ELECTROLYTES

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The observation that patients given arginine excrete increased quantities of potassium in the urine unrelated to nitrogen balance prompted the present investigation. Two possible explanations for this potassium diuresis seemed likely: (1) the displacement of extrarenal cellular potassium by arginine, as shown by in vitro experiments, and/or (2) an effect of arginine on the renal handling of electrolytes. The studies reported here were designed to investigate the latter possibility.

Four subjects were studied in a metabolic ward. L-Arginine, or L-glutamic acid, or L-arginine L-glutamate (155 mM.) was given daily for 3 days. The 3 days of amino acid administration were preceded and followed by control periods of from 6 to 12 days. Arginine (free base) and glutamic acid were given orally. Arginine hydrochloride, arginine glutamate, and sodium glutamate were administered intravenously. Daily urinary excretions of sodium, potassium, chloride, phosphorus, bicarbonate, ammonium, titratable acid, and pH were determined, and blood electroyltes were measured serially.

The administration of arginine, as the free amino acid orally, or as the hydrochloride or the glutamate salt intravenously, was consistently accompanied by increases in potassium excretion ranging from 10 to 65 per cent above control values. Arginine administration (except as the hydrochloride) was also accompanied by increases in urine pH and bicarbonate, and decreases in titratable acid and ammonium excretion. Even when arginine hydrochloride was given, acidification of the urine and ammonium production were delayed as compared to the renal response to hydrochloric acid. Alterations in blood electrolytes

occurred only with arginine hydrochloride which induced decreases in pH and carbon dioxide content.

Urinary potassium excretion was unaltered by sodium glutamate given intravenously or glutamic acid given orally. Increases in urine pH and a large excretion of bicarbonate occurred with sodium glutamate. Glutamic acid per se did not affect the blood or urine constituents measured. Glutamic acid given as arginine glutamate did not negate the effect of arginine on potassium excretion.

These data suggest that arginine, a cationic amino acid, enhances the urinary potassium excretion at the expense of hydrogen ion excretion. Similar alterations did not occur when glutamic acid, an anionic amino acid, was given.

54. ERYTHROCYTE DESTRUCTION IN THE LUNGS AS THE MAJOR CAUSE FOR ANEMIA IN PRIMARY PULMONARY HEMOSIDEROSIS

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Recent publications have denied or minimized a role for hemolysis in anemia of primary pulmonary hemosiderosis. Clinical observations and experimental studies conducted over 17 years in a man support the premise that a significant hemolytic mechanism exists in primary pulmonary hemosiderosis. His illness is characterized by episodes of anemia. The first one occurred at age 12 years, was sudden in onset, resulted in anemia with 4.1 Gm. of hemoglobin, but without hemoptysis. Seven subsequent attacks of anemia were accompanied by varying degrees of dyspnea, hemoptysis, and fever. Radiographic features of pulmonary hemosiderosis appeared. During acute illness, daily volume of hemoglobin-colored sputum ranged from a few to 250 ml. The anemia was severe. The lowest erythrocyte count was 900,000. Of the 38,000 ml. of blood transfused, 15,000 ml. was used in one hospitalization. The anemia could not be attributed to blood loss (hemoptysis) alone. Splenic sequestration was not a factor because after splenectomy was performed anemia recurred. Studies excluded lupus erythematosus, lymphoma, hepatic, heart, and parasitic disease, recognized hereditary hemolytic anemia of the hemoglobinopathy type, or diseases with red cell enzymatic defects. Bone marrow smears and reticulocyte values were consistent with increased erythropoiesis. Maximum marrow release of erythrocytes was calculated to be five times normal. In different phases of the illness, erythrocyte survival studies (Ashby method) were performed. During the acute illness, cell loss ranged from 3 to 11 per cent per day. During a recovery period, the loss rate changed from 3 to zero per cent. During remissions the erythrocyte survival was normal. Using Cr^{51}-tagged cells, the loss of erythrocytes was accelerated even during a period without hemoptysis. At this time, and subsequently, the highest concentration of radiochromium (as measured by collimated scintillation counter) was found to be over the right lung field. Fixed sections of hemoglobin containing sputum revealed few intact red cells but many macrophages with inclusion pigment.

The anemia of primary pulmonary hemosiderosis is fundamentally hemolytic in character with red cell destruction occurring within the lungs. Loss of intact erythrocytes by hemoptysis is ordinarily a minor factor.

55. HIGHER INCIDENCE OF PENICILLIN-RESISTANT STAPHYLOCOCCI ISOLATED FROM RHEUMATIC CHILDREN ON PENICILLIN PROPHYLAXIS THAN FROM CONTROL CHILDREN

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This study was undertaken in an attempt to shed light upon the question: "May nature be altered in a population of children to whom penicillin is administered over a period of years, so that a reservoir of penicillin-resistant staphylococci may develop in their noses and throats?" An obvious population which furnishes a ready source for study of this question is a local group of rheumatic children under continuous prophylactic management.

We are evaluating the status of penicillin resistance in staphylococci and certain biologic variations of these organisms, isolated from nose and throat cultures from rheumatic children treated with penicillin. Organisms similarly isolated from nontreated, apparently normal children in the same age group serve as controls.

From 135 rheumatic children treated with penicillin and from 80 nontreated nonrheumatic children, 284 cultures of staphylococci were isolated. Of these, 165 were obtained immediately before the children received their monthly injections of benzathine penicillin G at the Children's Cardiac Center. Isolated colonies were taken from Bacto-staphylococcus medium No. 110.

These organisms were tested for (1) production of coagulase, (2) production of pigment, (3) determination of hemolytic activity on blood agar, (4) fermentation of mannitol, and (5) resistance to penicillin in both high and low concentrations (10 and 2 units, respectively).

Results revealed (1) twofold higher incidence of penicillin-resistant strains from all *Staphylococcus aureus* (70 per cent) from the treated group in contrast to 32 per cent in the control group and (2) in nonpigmented (white) variants of *Staph. aureus* (coagulase positive), penicillin resistance was eightfold higher from treated children (17 per cent) in contrast to control children (2 per cent).

56. ELECTRON MICROSCOPIC EXAMINATION OF CELLS INFECTED WITH REOVIRUS

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Reoviruses were formerly classified as Echo type 10 but now are distinguished from enteroviruses mainly by their larger size and production of cytoplasmic inclusion bodies. The Dearing strain of reovirus was obtained from Dr. A. B. Sabin and was used to infect monkey kidney cells in tissue culture. Sections were prepared for electron microscopy by standard methods and were stained with uranium. It was found that the cytoplasm of many cells contained large crystalline masses of viruslike particles approximately 60 mμ in diameter as measured by the method of latex spheres. Study of internal structure of the particles showed mainly ring forms, some with double membranes and a few with dense inner bodies. The finding of crystalline cytoplasmic masses of particles confirmed the findings of Tournier, but our observations differed from his in that most of the particles in our preparations did not contain dense inner bodies. Therefore, since Tournier used KB cells, our observations were repeated

with this strain of cells. Under these conditions, it was found that most of the particles had dense inner bodies.

The results support the concept that growth of reoviruses occurs in the cytoplasm and suggest that enzymes necessary for viral synthesis are present or inducible in that location. The absence of dense inner bodies from most of the particles visualized in monkey kidney cells suggests that these structures are incomplete forms of virus and that the likelihood of their production is related to the type of cell infected by the virus. One may speculate that such forms lack nucleic acid and are not infectious but retain viral antigens. In addition, the findings give further indication that the electron microscopic examination of infected cells may provide information of value for the identification and classification of viruses that are isolated from patients.

57. RADIOCALCIUM METABOLISM IN HUMAN DISUSE OSTEOPOROSIS

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(INTRODUCED BY JOHN R. WALSH, M.D.)

Disuse osteoporosis is usually attributed to a decrease in osteoblastic activity secondary to reduced mechanical stress. Whereas this hypothesis seems to account for observations in immobilized, normal individuals, studies in paralyzed subjects have failed to show the expected effect of mechanical stress or its absence on calcium balance. In order to reinvestigate this problem, 15 combined balance and radiocalcium studies were performed in seven extensively paralyzed patients (poliomyelitis) and in four normal control subjects. Balances were determined over an average of 24 days and isotopic studies were followed up for at least 14 days.

During the developing phase of osteoporosis (strongly negative calcium balances) all patients showed a rate of long-term dilution of isotope significantly greater than that in normal subjects. Such dilution is produced primarily by dietary calcium absorption and by resorption of old (isotope-free) bone. Intestinal absorption was found to be less than normal in the subjects studied, and thus the greater rate of isotope dilution suggested increased bone resorption. Quantitative mathematical analysis indicated that total skeletal resorption was two to three times higher than in normal subjects.

Calculation of bone formation (based on analysis of isotope turnover and excretion) revealed rates varying from normal to twice normal (9 to 21 mg. calcium per kilogram per day). Subjects with long standing severe paralysis, but in calcium equilibrium, exhibited bone formation and destruction rates within normal limits (7.5 to 9.5 mg. calcium per kilogram per day).

Administration of norethandrolone to two paralyzed patients in the developing phase of osteoporosis produced the expected improvement in calcium balance, but was accompanied by no change in bone formation rates. Bone resorption rates, however, were markedly reduced and these reductions accounted entirely for the observed balance shifts.

These findings indicate that (1) development of disuse osteoporosis is related to gross elevations in bone destruction and not to reduction in new bone formation; (2) large scale reduction in skeletal stresses is not associated with detectable decreases in bone forming activity; (3) calcium balance effects of "anabolic" steroids may be due to an anticatabolic activity.

58. THE ANTIGENICITY OF A_2-HEMOGLOBIN

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The heterogeneity of normal adult human hemoglobin is a well-established fact. The present study was designed to determine if (a) the main fraction (A_1), the slow component (A_2), and the nonhemoglobin fraction (unidentified component 1, U.C.$_1$) have specific antigenicity, and (b) if A_2 can be demonstrated immunologically in cord blood and in the presence of S and C hemoglobin. Previously described techniques (Am. J. Clin. Path. 34: 28, 1960) were used for the preparation and agar gel electrophoresis of the hemolyzates and for the elution of the fractions from agar gel. Antisera to A_1, A_2, and U.C.$_1$ were prepared in rabbits. By an agar gel double diffusion technique and immunoelectrophoresis the antisera were tested against hemolyzates of normal adult, cord hemoglobin, hemoglobin S, A-S, A-C, S-thalassemia, and against separate eluates of portions of the agar gel electrophoretogram of normal, fetal, S, and A-C hemoglobins.

Antibody to A_2 hemoglobin appeared earlier than to A_1 and U.C.$_1$. A_1 was only weakly antigenic. With this technique A_2 and U.C.$_1$ were demonstrated in whole hemolyzates of hemoglobin A, S, A-S, C, A-C, and fetal. A_2 from normal, thalassemic, and cord blood were found to be immunologically identical. Anti-A_2 was immunologically specific and did not react with eluates of A_1 and the central portions of S, C, and fetal hemoglobin. It did, however, react with eluates from the most cathodic portions of S and fetal hemoglobin and with an eluate from the interspace between A and C hemoglobin.

The finding of specific antigenicity of A_2 hemoglobin is in agreement with the recently detected differences in the "finger print" between A_1 and A_2 hemoglobin. The presence of A_2 in hemoglobins other than A speaks for the specific genetic determination of this component.

59. EXPERIMENTAL PRODUCTION OF ATROPHIC GASTRITIS BY A PRESUMABLY IMMUNOLOGIC MECHANISM

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In 1959, Smith and co-workers reported that repeated intravenous injections of dialyzed, lyophilized human gastric juice produced reversible atrophic gastritis in dogs.

The present studies were undertaken to determine if similar lesions of the gastric mucosa could be produced by a presumably immunologic mechanism.

Eight dogs were studied. Each of the three experimental pairs of dogs received two injections intradermally of 4 mg. per kilogram of lyophilized, dialyzed, heterologous (human), isologous, or autologous gastric juice in Freund adjuvant at one month intervals. The control pair received adjuvant in saline. Surgical biopsies of the gastric fundus were made at monthly intervals.

At one month after the first injection, the dogs in each of the experimental pairs showed marked lysis of aprietal cells, the acellular spaces being filled with alcian blue–positive material. At 2 months, there was further loss of glandular

cells, eosinophilic and lymphocytic infiltration, and connective tissue condensation or proliferation. At 3 months a striking but partial atrophy of all glandular elements was seen together with proliferation of the superficial mucosa. At 4 to 5 months after the initial injection, reversal of the lesion was evident in some dogs. The histologic changes were as striking in the dogs which received their own gastric juice as in the other experimental groups. Histamine-fast achlorhydria was noted in some, but not all, of the experimental groups 3 months after the first injection.

Precipitins reacting with gastric juice were found in the experimental pairs. Positive delayed skin reactions with gastric juice were obtained in the experimental pairs only, at one month after the first injection. The control dogs showed none of these changes.

The relationship of this lesion to the gastric atrophy produced by intravenous injections of human gastric juice is not yet clear. It appears, however, from this present study that gastric atrophy can be produced by an immunologic mechanism. The ease with which this lesion can be produced by autologous gastric juice makes it important to consider the possibility that similar mechanisms might be involved in diseases of man associated with atrophic gastritis.

60. STUDIES OF ADIPOSE TISSUE AND LIVER GLYCOGEN IN RATS LIMITED TO A SHORT DAILY FEEDING PERIOD

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(Introduced by A. J. Bollet, M.D.)

Many obese people consume most of their food within a short period each day. Stunkard used the term "night eating syndrome" to describe a group of obese patients whose major food intake occurred during the evening hours and suggested that this eating pattern was related to psychologic disturbances.

We have studied rats allowed access to food for only 2 hours of each 24 for periods of 1 to 7 days in an effort to determine what adaptive changes take place when food intake is manipulated in this way. Groups of five or six rats were studied at the end of the feeding period after 1 to 7 days on this feeding program with these findings: (1) food intake during the 2 hour period remained relatively constant over 7 days and body weight decreased slightly; (2) in vitro incorporation of C^{14} acetate into lipids by adipose tissue increased over thirtyfold within the period of study while incorporation of C^{14} into liver lipids increased only fourfold; (3) galactose-6-phosphate and 6-G-P dehydrogenase activity in adipose tissue rose about 400 per cent but did not change in liver; (4) liver glycogen increased during the first 3 days of the study and remained relatively constant thereafter; (5) the free fatty acid content of adipose tissue fell gradually during the period of study; (6) liver glycogen in animals limited to a daily feeding period of 2 hours for 4 or 5 days was much greater after 24 hours of fasting than fasted controls who had been allowed to eat ad libitum.

These remarkable adaptive changes stimulate new interest in the disordered eating patterns observed in clinical obesity. A major metabolic consequence of limited feeding periods appears to be greatly increased fat synthesis which is self-perpetuating.

61. STUDIES ON THE NATURE OF THE LIPID DISTURBANCE IN CYSTIC FIBROSIS OF THE PANCREAS

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Cystic fibrosis is a generalized disease of exocrine glands which manifests itself clinically by loss or diminution of pancreatic exocrine secretions, by increased susceptibility to upper respiratory infections, and by elevation of sodium and chloride concentrations in the sweat. The recent demonstration by Holman and co-workers (Pediatrics 24: 34, 1959) of a decrease of aortic atherosclerosis in patients with this condition made it essential that a systemic investigation be undertaken on the nature of the lipid disturbance in cystic fibrosis of the pancreas.

Serum was obtained after an overnight fast in a group of fibrocystic children and comparable normal control subjects. Analysis for cholesterol, phospholipids, and triglycerides showed no significant difference between the two groups. Ultracentrifugal analysis of the serum, however, revealed a decrease of the Sf 0-10 fraction and a decrease of the "atherogenic index." These findings are opposite to those usually seen in idiopathic hyperlipemia and primary hypercholesteremia.

The children were then given a breakfast containing 90 Gm. of fat and serum samples were taken before and 4 hours after the meal. It was found that the fibrocystic children showed a decreased ability to reabsorb triglycerides as compared with the normal children, whereas the phospholipid and cholesterol levels were essentially the same. This abnormality may be attributed to a deficiency of pancreatic lipase.

However, this defect should not interfere with the absorption and utilization of fat administered intravenously. Accordingly, 1 Gm. per kilogram body weight of Lipomul was given intravenously. It was found that whereas normal children showed a good clearing of the serum by 4 hours, the serum from fibrocystic children remained turbid. Furthermore, previous preparation with 1 mg. per kilogram body weight of heparin failed to clear the hyperlipemia. This suggests that there may exist a second defect that involves lipoprotein lipase in cystic fibrosis. This was confirmed by finding a marked decrease or complete absence of this enzyme in the affected children.

62. DETERMINATION OF CONSTRICTOR SUBSTANCE IN PLASMA BY MEANS OF A STRIP OF RABBIT AORTA AS AN AID IN THE DIFFERENTIAL DIAGNOSIS OF RENAL VASCULAR OCCLUSIVE DISEASE

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Spirally cut strips of rabbit thoracic aorta have proved to be a useful preparation for the demonstration of vasoconstrictor substances in plasma of patients with arterial hypertension (Hypertension 8: 38, 1959). Samples of blood have been obtained by catheterization from the right and left renal veins from seven patients with documented hypertension secondary to renal arterial obstructive disease. In all cases, 0.5 to 2.0 ml. of dialyzed plasma from the renal vein of the involved kidneys caused a strong contraction of the strip. Plasmas from the uninvolved kidneys showed much less or minimal vasoactivity. When constrictor activity was present in renal venous plasma, it

could also be detected in peripheral arterial and venous blood. After nephrectomy or reconstructive renal vascular surgical operation, blood pressure fell to normal or near normal levels. Samples of plasmas obtained as early as 6 hours and as late as 1 year after surgical operation possessed no demonstrable constrictor or pressor activity.

In addition to the assay for vasoconstrictor activity, intravenous and retrograde urograms, differential renal function measurements, renal angiograms, and determinations of cardiac output (Fick) were done. In all patients retrograde pyelography disclosed no structural abnormalities in the pelviocalyceal system. In three of the patients, intravenous urograms were normal. Except for two patients, one with bilateral renal arterial constriction and the other with a "branch" occlusive renal arterial lesion, differential renal functional measurements demonstrated decreases in the function of the affected kidney. Renal angiograms showed the type and distribution of the occlusive renal arterial lesion in every case. Except for one patient in congestive heart failure, the cardiac output determinations were within the normal range.

The assay of peripheral venous blood for vasoconstrictor activity may prove to be a valuable diagnostic screening test for patients with remedial hypertension caused by renal vascular occlusive disease.

63. HEPATO-TOXIC EFFECT OF NEWER ANDROGENS

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In evaluation of oral androgenic compounds in the treatment of advanced breast cancer, investigations of liver function have revealed a disparity in the type, frequency, and rapidity of onset of the biochemical findings indicating liver toxicity. Jaundice and laboratory findings of biliary obstruction have occasionally been reported with methyltestosterone, more frequently with 17-ethyl-19-nortestosterone, and rarely with fluoxymesterone. The abnormality has occurred after prolonged therapy. A very high incidence of sulfobromophthalein retention and serum bilirubin elevation has been reported with 17a-methyl-19-nortestosterone.

Investigations revealed that 17a-methyl-19-nortestosterone produced persistent elevation of serum bilirubin and sulfobromophthalein retention when therapy was extended beyond 6 weeks. The degree of liver function prohibited long-term use of the compound for cancer therapy.

2a-17a-Methyl dihydrotestosterone was administered to five patients with advanced breast cancer. There was an abrupt increase in sulfobromophthalein retention and marked increase in serum glutamic oxalacetic transaminase and glutamic pyruvate transaminase within one week of therapy. The serum bilirubin became elevated if therapy was continued. No elevation of serum alkaline phosphatase occurred. Other liver function tests remained normal. The abnormalities reverted to normal a few weeks after therapy was stopped.

The disparity of biochemical findings of liver function suggests that the chemical structure and characteristics of the androgen may be related to the sequence of signs of hepato-toxicity. With 2a-17a-methyl dihydrotestosterone the initial effect may be within the liver cell.

64. EFFECTS IN DOGS OF A LARGE WATER LOAD UPON THE VOLUME AND COMPOSITION OF EXTRACELLULAR FLUID

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(INTRODUCED BY J. C. PETERSON, M.D.)

In 1954, Leaf found the volume of distribution of a water load equal to that of deuterium. Brodsky, in 1957, found the solute concentration of tissue equal to serum. Leaf confirmed this in 1958. These findings lead one to presume that the cellular compartment accepts a proportionate share of a water load.

We have anesthetized dogs with pentobarbital, tied the renal veins, arteries, and ureters, and then primed the animals with inulin and bromide. After 2 hours distilled water equivalent to 15 per cent of body water volume was given rapidly intravenously. Muscle was biopsied before and after water loading. Five dogs received tracer doses of Na^{22} 24 hours before the experiment.

Four hours after the start of the water load inulin and bromide space had each increased by 30 per cent of initial volume. Total chloride of inulin and bromide space was unchanged. Each space had gained 1.7 mEq. sodium, 100 ml. of initial volume. Total exchangeable sodium was unchanged. Significant loss of muscle cell sodium occurred. Blood pH was measured before and after water loading. Mobilization of sodium from muscle was not dependent upon a fall in pH.

The findings indicate that dilutional hyponatremia may mobilize sodium from muscle cells. Mobile sodium may then obligate water extracellularly. The ECW volume then determining the distribution of a water load will be greater than the initial ECW volume. Relative to initial volumes, the increase in ECW after water loading may, therefore, be greater than the increase in total body water. In this way a water load may be largely confined to extracellular fluid. Thus, although the volume of distribution of a water load be total body water, the cellular compartment need not accept a proportionate share of the load.